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13. ABSTRACT *(Maximum 200 Words)*

This project studies interactions between genes, environment, and age in causing mouse Parkinsonism. We use mice overexpressing wild-type or a doubly mutated form of human α–synuclein (hα-SYN). We created and characterized these two constructs on DNA, RNA, and protein levels. Both the wild-type and doubly mutated lines express functional hα-SYN in dopaminergic terminals as demonstrated by altered locomotor responses to systemic amphetamine and overexpression of the dopamine transporter. Both constructs are more sensitive to acute doses of MPTP. The doubly-mutated hα-SYN line has altered levels of motor behavior and altered levels of dopamine (DA) and metabolites throughout its age span. At a young age, this line is hyperactive, progressing to normal levels of activity in middle age, but becoming hypoactive at an old age. These changes are paralleled by alterations in DA and metabolites and resemble those seen in human Parkinsonism. Finally, the doubly-mutated hα-SYN line is very sensitive to the combination of systemic neurotoxicants, maneb and paraquat demonstrating significant reductions in locomotor activity, DA, and substantia nigra neuron number. We intend to continue our aging and neurotoxicant studies in the next year and establish our somatic mosaic lines to study the role of selective aggregation as a mechanism of toxicity.

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Eric Rushfield 9/27/01

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INTRODUCTION

The subject of this research is to understand the role of genes, aging, and neurotoxicants on the etiology of Parkinson's disease (PD). One specific gene, the human α-synuclein gene, was selected for study because of its role in a familial form of Parkinson's disease. Recent evidence also implicates α-synuclein in idiopathic or other forms of Parkinson's disease. The presence of the α-synuclein protein in Lewy bodies and dystrophic neurites has been found. In addition, polymorphisms or unique haplotypes exist in some patients with sporadic Parkinson's disease suggesting that dysregulation of this gene and its wild-type protein may play a role in idiopathic Parkinson's disease. The other major risk factor for Parkinson's disease is environmental exposure and neurotoxicants. MPTP is a known cause of a form of Parkinsonism. It has been speculated, based on epidemiologic studies and animal studies, that other environmental agents may predispose to sporadic forms of Parkinson's disease. Recent studies in our lab and other labs have suggested that paraquat may be selective dopaminergic neurotoxicant. In addition, the environmental dithiocarbarmate pesticides have been found to augment toxicity of both MPTP and paraquat.

The purpose of this research is to develop appropriate models, to study the interactions of α-synuclein, and neurotoxicants. These models will demonstrate whether dysfunction and death occur in mouse models and how it may be prevented. Our primary method of investigation is the use of transgenic mice, expressing either the wild type human α-synuclein or a doubly mutated form of α-synuclein. These mice are studied in the context of normal aging and following exposure to neurotoxicants.

BODY

Our application stated that environmental toxins alone or genetic mutations alone might produce forms of Parkinson's disease. We also hypothesized that sporadic cases of Parkinson's disease may be the result of interactions between three factors including genetic predisposition, aging, and either life long exposures to neurotoxicants or acute intermittent exposure to neurotoxicants. We hypothesized that: (1) a mutated form of human α-synuclein would be toxic to substantia nigra neurons via a toxic gain of function when over expressed in the mouse and (2) that over expression of either the mutated α-synuclein gene or the wild type α-synuclein gene would contribute to increased vulnerability to environmental neurotoxicants in mouse substantia nigra. We finally hypothesized that aging would play an important role in the interaction of human α-synuclein and neurotoxicants in contributing to Parkinson's disease.

The third year of this grant has been our most successful year in terms of abstracts and manuscripts. But more importantly, the results we have obtained have demonstrated that most of our hypotheses will be born out. Our only disappointment at this point is the lack of publications. However, we expect once our first manuscript is accepted we will have a series of manuscripts over the next year as a result of the efforts we have been making. Data and plans for those papers will be presented in the context of this report.

We will summarize our work in relationship to our most recent SOW. We will also include data on other related projects we are involved in that relate to models of PD.

Years 1 and 2. Data from Years 1 and 2 have been presented in the previous annual reports. Specific work completed in Years 1 and 2 includes the following:

Ia. Transgenic mouse lines over expressing wild type or double mutated human α-synuclein under control of TH promotor have been completed.

IIb. Reagents and characterization of both constructs at the DNA, RNA and protein level has been completed.

Ic. Assessment of these constructs for functionality and response to neurotoxicants using behavioral, neurochemical and stereological measures. This has been completed in part. Some of this data is presented in our manuscript entitled "Physiologic and pathologic effects of wild-type and mutated human α-synuclein in transgenic mice" included in the appendix. Information in this manuscript will be discussed under Year 3, as some of that work was performed in Year 3 and is included in that manuscript.

Id. Create a universal somatic mosaic vector for the production of somatic mosaic lines and test all functional DNA elements. This has been completed.

Year 2

IIa. Two α-synuclein XAT constructs will be made. *In vitro* testing of the constructs using transfected cells. We have cloned the α-synuclein gene into our pUSMTV along with the human prion promotor. All clones to date have been in the wrong orientation. We are redesigning our PCR primers to ensure correct orientation.

IIc. Generation of transgenic mice. This task has not yet been completed, due to efforts spent in Year 3 on other studies to be described using the lines already created. Most of the hypotheses we made can be tested using standard transgenic models as we have done and will continue to do. However, we expect cloned XAT constructs soon. We will finish sequencing of the constructs so that they can be sent to our transgenic core for generation of mice. Elements of the universal somatic mosaic cloning vector have previously been tested. Minimal testing for expression using transcient transfection will be performed prior to generation of animals.

IId. Begin testing for aging and neurotoxicant effects using the transgenic lines. Significant work in this area has been accomplished and will be discussed in detail in Year 3 summary.

Year 3.

Ic. In order to access the functionality of the α-synuclein lines already generated, previous reviewers have requested us to explore effect of amphetamine on locomotor activity. In combination with testing additional lines of mice, we have performed two different studies on the effect of amphetamine in these transgenic lines. In the first experiment described in our manuscript (appendix), we demonstrate that a second low dose of amphetamine resulted in a significant increase in locomotor activity in a wild-type transgenic line (hwα-SYN-5) and significantly reduced locomotor activity in the double mutant line (hm^2α-SYN-39), comparison to non-transgenic littermates. This suggested to us that α-synuclein was functional in our transgenic lines and was having a specific effect in dopaminergic terminals. The increased activity in the wild-type line suggested that the excess human α-synuclein was functioning and augmented the biochemical response to amphetamine. The reduced response in the double mutant line suggested that the double mutant α-synuclein might be impairing the activity of endogenous mouse α-synuclein and also suggested that α-synuclein may play an important role in mediating dopaminergic response to amphetamine. We repeated this study with modifications using two additional transgenic lines. We modified the regimen to use a higher dose of amphetamine given biweekly and to have a one-week absence prior to the last of seventh dose. Using this paradigm, we were able to demonstrate true sensitization to amphetamine in both non-transgenic controls and in a wild-type line (hwα-SYN-88). However, the double mutant line (hm^2α-SYN-10) failed to demonstrate sensitization. This again suggests that the double mutant form of synuclein is impairing dopamine terminals and their response to repeated amphetamine. This suggests that mutant α-synuclein may act by a loss of function in the endogenous mouse α-synuclein. It also demonstrates a functional role for α-synuclein in sensitization to amphetamine.

IId, IIIb, IIIc, and IVb. We have obtained data on our first approach to the effect of aging as an interaction with α-synuclein. This data is presented in part in our manuscript. In that paper, we demonstrate that young mice with the double mutant form of synuclein (line hm^2α-SYN-39) are abnormal at an early age, as manifested by increased locomotor activity and a reduced (faster) time in a motor task compared to either nontransgenic littermate controls or a wild-type line (hwα-SYN-5). Associated with these increased functions were reduced levels of dopamine and abnormal dopamine metabolites. As that line aged, it went through a transition where the locomotor activity was comparable to non-transgenic littermates and line hwα-SYN-5 and continued to progress at a older age where they had significantly reduced locomotor activity and a significantly increased (slower) time with a motor screen. These motor effects are similar to those seen in the human condition of Parkinson's disease known as bradykinesia. As they aged, they also had progressive changes in dopamine and dopamine metabolites. This suggests that the

double mutant form of α-synuclein is adverse on its own beginning at a young age and continuing throughout the life span of the animal, eventually resulting in a Parkinsonian phenotype. These studies were done as multiple cross sectional studies with mice selected at each of the age groups (young-age, middle-aged, and old-age). We have a longitudinal study studying the same mice over time in which we measure the same motor tasks. This study is halfway through, having tested the young- and middle-aged time points. We are now waiting for them to become older. This data will be useful for us to map the specific rate of motor decline with time and will be useful for designing interventions or treatments to alter the course of this physiologic decline. As the mice age and they develop significant changes in motor function, we will also begin testing these animals to see if they respond to typical dopaminergic treatments for Parkinson's disease, such as the use of L-dopa or direct dopamine agonists. We will then be able to correlate functional changes with a variety of biochemical and neuronal outcomes.

We are also in the process of completing our stereologic counts of substantia nigra pars compacta neuron number with aging. We have completed counts that the middle aged group (see Table below) and are in the process of completing counts in the younger and older age groups to demonstrate whether the functional and biochemical effects are also associated with neuronal loss. We expect to have this data completed shortly.

We plan to submit a manuscript documenting these age-related declines in function and neuron counts and the reversal of functional decline with dopaminergic agonists. We feel that in terms of the aging study we are significantly ahead of our time table and this will allow us time to study a variety of consequences and mechanisms related to this neuronal loss. We feel this model may be an ideal age-related model of parkinsonism in mice.

Ic, IId, IIIb, and IVb. We have made considerable progress in demonstrating an interaction between α-synuclein and neurotoxicants. We have completed one study, which is in final preparation and should be ready for submission soon. In this study we treated non-transgenic controls, lines hwα-SYN-5 and hm^{2}α-SYN-39 with chronic maneb and paraquat. As reported previously, we have been involved in studies with Dr. Deborah Cory-Slechta on her neurotoxicant model of Parkinson's disease and have taken advantage of that work using the transgenic mice (see Appendix). We performed behavioral measures, neurochemical measures, and stereologic measures, which are illustrated below (Figures 1 and 2 and Table). In this study we used half the normal doses of maneb and paraquat that we normally use in non-transgenic littermates because line hm^{2}α-SYN-39 was too sensitive. Despite this lower dose, line hm^2-39 was affected with highly significant losses in locomotor activity (**Fig 1**), associated with significant losses in dopamine and dopamine metabolites (**Fig 2**) and significant losses in TH positive neurons in the substantia nigra pars compacta (**Table**).

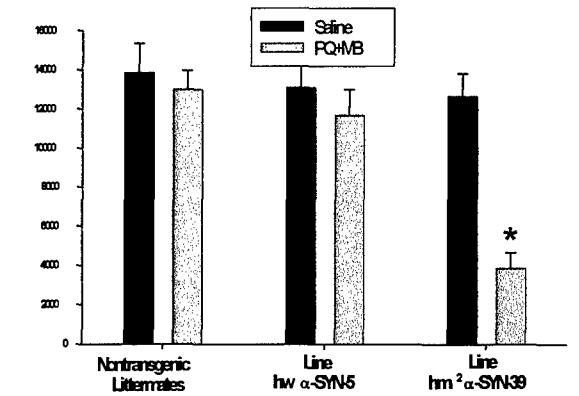

Fig 1. Behavior was assessed immediately after dosing, 24 hrs later, or 10 days after the last dose to assess acute effects, immediate recovery and permanent effects. This figure shows final locomotor behavior 10 days following the last dose of saline or combined PQ+MB. *PQ+MB resulted in a permanent and significant decline ($p < 0.0001$) in locomotor activity **only** in line hm^{2}α-SYN-39.

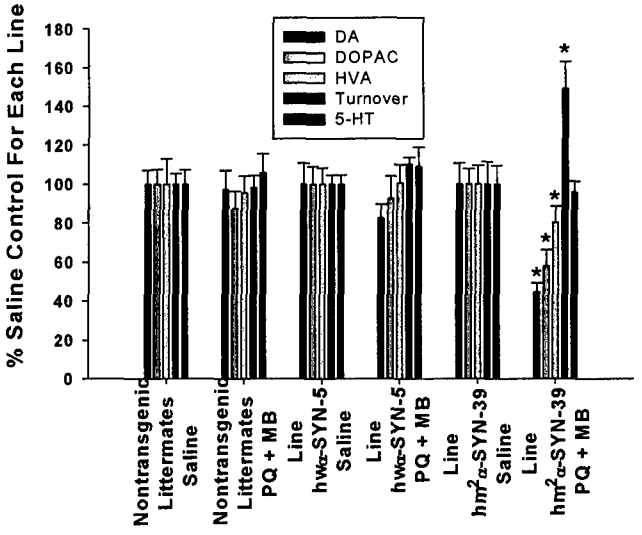

Fig 2. DA and metabolite values were normalized to the saline control for each line. This allows the data to be visualized on one plot at the same scale. Values for the nontransgenic littermates treated with saline are as follows (ng/mg protein ± SEM): DA (106.6 ± 7.6), DOPAC (10.1 ± 0.8), HVA (13.4 ± 1.9), DA turnover (16.0 ± 0.9), 5-HT (5.8 ± 0.4). Values indicated by * for PQ+MB are significantly ($p < 0.005$) different from their saline controls.

Mouse line	Treatment	n	TH+ Nest	% saline	TH- Nest	Total Neuron (calculated)
Nontransgenic Littermates	Saline	3	8,213 ± 308		4,467 ± 254	12,680 ± 533
Nontransgenic Littermates	PQ+MB	3	**6,507 ± 278**[a]	79%	4,493 ± 53	11,000 ± 323
Line hwα-SYN-5	Saline	3	8,507 ± 278		4,860 ± 89	13,346 ± 213
Line hwα-SYN-5	PQ+MB	3	**7,066 ±116**[a]	83%	4,461 ± 94	11,666 ± 232
Line hm²α-SYN-39	Saline	3	6,213 ±141		3,933 ± 71	10,146 ± 187
Line hm²α-SYN-39	PQ+MB	3	**2,991 ± 70**[a]	**48%**[b]	4,116 ± 607	7,107 ± 616

Table. Stereologic counts of mouse SNpc neurons in the three lines following saline or PQ+MB. Values marked by [a] indicate significant difference following PQ+MB versus saline ($p < 0.0001$) and by [b] indicate significant difference ($p < 0.0001$) from nontransgenic littermates and line hwα-SYN-5. Note that combined PQ+MB produced equal and significant neuronal loss of neurons in nontransgenic littermates and line hwα-SYN-5 without changes in behavior, DA, or DA metabolites. This is consistent with our previous work. Note also that line hm²α-SYN-39 had significantly fewer neurons at middle age than the other two groups. Whether this represents a loss at this age will not be known until we complete our neuronal counts at the younger age.

This is the first demonstration of a specific interaction between a known genetic mutation and environmental neurotoxicants supporting a hypothesis of such an interaction.

We have also begun our studies using acute MPTP. In our first study presented in our manuscript, we used only two very low doses of MPTP separated by one week. We saw significant changes in both transgenic lines suggesting increased vulnerability to this neurotoxicant. We are in the process of repeating this study using larger and more frequent doses to see if we can demonstrate increased vulnerability in our double mutant lines compared to the wild-type line. We believe this study is likely to show that over expression of wild-type synuclein may be adverse in terms of increased vulnerability to some neurotoxicants, but the mutant form is likely to be much more adverse. We will be using the same paradigm as we used for paraquat and maneb described above in which we measure behavior, biochemistry, and neuronal counts. In this study, we are going to also test our other transgenic lines to verify that the defect is not restricted to one line. We expect to complete this study in the next year as it is an acute study and all of the outcome measures have been established.

We will also do the neurotoxicant studies at different ages to demonstrate the degree to which aging adds to toxicity.

KEY RESEARCH ACCOMPLISHMENTS IN YEAR 3

1. Resubmission of a manuscript entitled "Physiologic and pathologic effects of wild-type and mutated human α-synuclein in transgenic mice" (see Appendix).

2. Publication of a paper entitled "The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and Maneb: Implications for Parkinson's disease." Journal of Neuroscience 20:9207-9214 (see Appendix).

3. Submission of a manuscript entitled "Fluorescence stereology: A novel method to count neurons."

4. Identification of specific interactions of α-synuclein with the dopamine transporter and other functional effects in dopamine terminals. The adverse effects of the double mutant form of α-synuclein in two different transgenic lines suggest this may be an important mechanism underlying its role in Parkinson's disease.

5. Identification of aging-related behavioral and biochemical effects in the double mutant form. A longitudinal study is underway.

6. Cloning of α-synuclein into the universal somatic mosaic transgenic vector.

7. Development of stereology for neuronal counts of aging and neurotoxicant treated mice.

REPORTABLE OUTCOMES

Manuscripts Accepted

Thiruchelvam, M., Brockel, B.J., Richfield, E.K., Baggs, R., Cory-Slechta, D.A. (2000) Potentiated and preferential effects of combined paraquant and maneb on nigrostriatal dopamine systems: Environmental risk factors for Parkinson's disease? *Brain Research* 873:225-234.

Thiruchelvam, M., Richfield, E.K., Baggs, R., Tank, A.W., and, Cory-Slechta, D.A. (2000) The nigrostriatal dopaminergic system as a preferential target of repeated exposures to combined paraquat and Maneb: Implications for Parkinson's disease. *Journal of Neuroscience* 20:9207-9214.

Richfield, E.K. and Farrer, M. (2001) Genetic risk factors in Parkinson's disease. NeuroToxicology 22: (in press)

Manuscripts Submitted

Richfield, E.K., Cory-Slechta, D.A., Thiruchelvan, M.J., Wuertzer, C.A., Gainetdinov. R., Caron, M., and Federoff, H.J. Physiologic and pathologic effects of wild-type and mutated human α-synuclein in transgenic mice.

Thiruchelvam, M., Federoff, H.J., Cory-Slechta, D.A., Richfield, E.K. Gene-Environment Interaction in Parkinson's Disease: Combined Exposure to Paraquat (PQ) and Maneb (MB) in Transgenic Mice Expressing Human Wild-Type or Doubly Mutated α–Synuclein.

Manuscripts in Preparation

Abstracts

Thiruchelvam, M., Richfield, E.K., Baggs, R., Tank, A.W., and, Cory-Slechta, D.A. Chronic exposure to paraquat and maneb: Effects on nigrostriatal dopaminergic system and implications for Parkinson's disease *Society for Neuroscience Abst.*, 2000.

Richfield, E.K., Cory-Slechta, D.A., Thiruchelvan, M.J., Gainetdinov. R., Caron, M., and Federoff, H.J. Altered dopamine terminals and enhanced vulnerability to MPTP in wild-type and mutated human α-synuclein transgenic mice. *Society for Neuroscience Abst.*, 2000.

Thiruchelvam M, Richfield EK, Tank AW, Baggs R, McCormack AL, Di Monte DA, Cory-Slechta DA (2001). Age related and irreversible nigrostriatal dopamine system neurotoxicity of combined exposure to paraquat and maneb. The Toxicologist, Vol 60 (1), p.54.

Cory-Slechta DA, Thiruchelvam M, Richfield EK, Thiffault C, Di Monte DA (2001). Age-dependent and progressive nigrostriatal dopaminergic neurotoxicity of paraquat. The Toxicologist, Vol 60 (1), p.54.

Barlow, BK, Thiruchelvam, M, Cory-Slechta, DA, and Richfield EK. *In vitro* augmentation of dopamine uptake by fungicides. *NTX XIX: Nineteenth International Neurotoxicology Conference* (2001).

Richfield, E.K., Thiruchelvam, M., Wuertzer, C.A., Cory-Slechta, D.A, and Federoff, H.J. Physiologic and pathologic features of wild-type and mutated human α-synuclein in transgenic mice. *NTX XIX: Nineteenth International Neurotoxicology Conference* (2001).

Thiruchelvam, M., Richfield, E.K., Federoff, H.J. and, Cory-Slechta, D.A. Effect of combined exposure to paraquat and maneb in transgenic mice expressing human wild-type or doubly mutant α-synuclein: Gene-environment interactions in Parkinson's disease. *Society for Neuroscience Abst.*, 2001.

Richfield, E.K., Thiruchelvam, M., Wuertzer, C.A., Cory-Slechta, D.A, and Federoff, H.J. Physiologic and pathologic features of wild-type and mutated human α-synuclein in transgenic mice. *Society for Neuroscience Abst.*, 2001.

CONCLUSIONS

We have made considerable progress in the third year of this award. We have demonstrated significant effects of both the wild-type and double mutant form *in vivo* in transgenic mice. It is important to emphasize that this is one of the few papers in which a transgenic human protein has been demonstrated to have a functional effect related to its likely normal action in man and mouse. We also demonstrate pathologic consequences of this expression for both the wild-type and double mutant form, but the double mutant form has significantly more adverse impact that increases with aging. Vulnerability to specific neurotoxicants was also demonstrated. We now have additional data demonstrating that the effect is not unique to one line, but occurs in several lines.

REFERENCES

None.

APPENDICES

1. Reprint of Journal of Neuroscience manuscript.

2. Copy of manuscript under review entitled "Physiologic and pathologic effects of wild-type and mutated human α-synuclein and transgenic mice."

3. Copies of Abstracts presented at recent meetings.

The Journal of Neuroscience, December 15, 2000, 20(24):9207–9214

The Nigrostriatal Dopaminergic System as a Preferential Target of Repeated Exposures to Combined Paraquat and Maneb: Implications for Parkinson's Disease

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Experimental evidence supporting 1,1′-dimethyl-4,4′-bipyridinium [paraquat (PQ)] as a risk factor for Parkinson's disease (PD) is equivocal. Other agricultural chemicals, including dithiocarbamate fungicides such as manganese ethylenebisdithiocarbamate [maneb (MB)], are widely used in the same geographical regions as paraquat and also impact dopamine systems, suggesting that mixtures may be more relevant etiological models. This study therefore proposed that combined PQ and MB exposures would produce greater effects on dopamine (DA) systems than would either compound administered alone. Male C57BL/6 mice were treated twice a week for 6 weeks with intraperitoneal saline, 10 mg/kg paraquat, 30 mg/kg maneb, or their combination (PQ + MB). MB, but not PQ, reduced motor activity immediately after treatment, and this effect was potentiated by combined PQ + MB treatment. As treatments progressed, only the combined PQ + MB group evidenced a failure of motor activity levels to recover within 24 hr. Striatal DA and dihydroxyphenylacetic acid increased 1–3 d and decreased 7 d after injections. Only PQ + MB reduced tyrosine hydroxylase (TH) and DA transporter immunoreactivity and did so in dorsal striatum but not nucleus accumbens. Correspondingly, striatal TH protein levels were decreased only by combined PQ + MB 5 d after injection. Reactive gliosis occurred only in response to combined PQ + MB in dorsal–medial but not ventral striatum. TH immunoreactivity and cell counts were reduced only by PQ + MB and in the substantia nigra but not ventral tegmental area. These synergistic effects of combined PQ + MB, preferentially expressed in the nigrostriatal DA system, suggest that such mixtures could play a role in the etiology of PD.

Key words: dopamine; striatum; nucleus accumbens; substantia nigra; tyrosine hydroxylase; dopamine transporter; locomotor activity; gliosis

Parkinson's disease (PD), a profound movement disorder resulting from nigrostriatal dopaminergic (DA) system degeneration, has been linked to living in a rural environment, farming, drinking well water, and occupational exposure to agricultural chemicals, suggesting an environmental exposure basis for the disease (Rajput et al., 1987; Semchuk et al., 1992; Liou et al., 1997; Gorell et al., 1998; Tanner et al., 1999). Correspondingly, a recent comprehensive study of over 19,000 white male twins showed that genetic heritability is not the basis of sporadic PD with onset over age 50 (Tanner et al., 1999). The identification of MPTP, a synthetic heroin that destroys substantia nigra DA neurons, has given additional credence to an environmental factor hypothesis (Langston et al., 1984; Langston and Irwin, 1986; Tanner and Ben-Shlomo, 1999). MPTP, however, is not found in the environment.

The herbicide 1,1′-dimethyl-4,4′-bipyridinium [paraquat (PQ)] has emerged as a putative risk factor on the basis of its structural homology to MPP^+, the active metabolite of MPTP. Occupational PQ exposures have been associated with parkinsonism (Hertzman et al., 1990; Liou et al., 1997). Although substantially impeded, PQ does cross the blood–brain barrier, with higher levels evident 24 hr as compared with 30 min after administration (Widdowson et al., 1996a).

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PQ, however, is a member of only one class of agricultural chemicals known to impact DA systems adversely and to be used in overlapping geographical areas. Diethyldithiocarbamate fungicides potentiate MPTP neurotoxicity (Miller et al., 1991; Walters et al., 1999). Manganese ethylenebisdithiocarbamate [maneb (MB)], for example, decreases locomotor activity (Morato et al., 1989) and potentiates MPTP effects on locomotor activity and catalepsy (Takahashi et al., 1989). Interestingly, at least two incidences of parkinsonism in humans have been related to MB exposure (Ferraz et al., 1988; Meco et al., 1994). The heaviest use of both PQ and diethyldithiocarbamates like MB occurs along the Pacific Coast and in the Northeast, the Plains, the mid-Atlantic, the Southeast states, and Texas (United States Geographic Service, 1998). PQ and diethyldithiocarbamates are also used on many of the same crops [e.g., tomatoes (Wilhoit et al., 1999)]. The geographical overlap in use patterns and the fact that multiple pesticide residues can be found in foods suggest that human exposures are to agrichemical mixtures, raising the possibility of multiple-hit models of PD. In such models, exposure to an individual chemical may be insufficient to induce overt effects, whereas multiple concurrent exposures, by provoking changes at multiple target sites of the nigrostriatal DA system, could preclude the operation of homeostatic defense mechanisms leading to neuronal cell death.

On the basis of this premise, we examined the hypothesis that combined PQ and MB (PQ + MB) exposures would produce additive effects on the nigrostriatal DA system. A previous experiment from our laboratory provided initial but limited support for this assertion (Thiruchelvam et al., 2000). Combined PQ + MB administered to mice once a week for 4 weeks potentiated reductions in locomotor activity and increased striatal DA, its metabolites, and DA turnover. It also reduced tyrosine hydroxylase (TH) density relative to either compound administered alone and did so in the dorsal striatum but not the nucleus accumbens, suggesting a

targeting of the nigrostriatal DA system. The current study sought to validate this model and to determine the selectivity and sensitivity of combined PQ + MB for the nigrostriatal system, the impact of exposure duration, and the involvement of substantia nigra DA cell bodies. Collectively the findings are consistent with the conclusions that combined PQ + MB exposure is preferentially neurotoxic to the nigrostriatal DA system, that this effect occurs via enhanced DA activity, and that such combined exposures may serve as environmental risk factors for PD.

MATERIALS AND METHODS

Animals

Male C57BL/6 mice 6 weeks of age purchased from Taconic (Germantown, NY) were housed in a room maintained under constant temperature (72–74°F) and humidity conditions with a 12/12 hr light/dark cycle. Five-month-old male C57BL/6 mice used in the studies to determine the extent and nature of neurotoxicity associated with combined PQ and MB were purchased from the National Institute on Aging aging colony (Harlan Sprague Dawley, Indianapolis, IN). Those used for behavioral studies were housed one per cage; all other mice were housed five per cage. Food and water were available *ad libitum*. Mice were habituated to the vivarium for at least 1 week before commencement of experiments. Body weights were obtained daily over the course of the experiment. Animals were cared for and treated in accord with National Institutes of Health and the University of Rochester Animal Care and Use Committee guidelines.

Chemicals

Solvents for HPLC with electrochemical detection (HPLC-EC) were purchased from Sigma (St. Louis, MO). All other chemicals, if not specified, were at least analytical grade and were purchased from Sigma.

Drug administration

Mice were injected intraperitoneally with either saline (vehicle), 10 mg/kg paraquat dichloride hydrate (Sigma), 30 mg/kg maneb (gift from DuPont Agricultural Products), or PQ + MB. A range of LD_{50} values for PQ has been reported for mice, extending from 30 to 60–70 mg/kg and suggesting that strain, gender, and solution factors are important (Bus et al., 1976; Drew and Gram, 1979; Yamamoto, 1993). Corresponding values for MB in mice are not reported, although LD_{50} values for oral administration in rats range from 4.5 to 6.7 gm/kg (Berg, 1977). Both PQ and MB were dissolved in saline. Mice were injected twice a week for 6 weeks for a total of 12 injections. For combined injections, two separate injections were administered. Animals were killed at varying time points, ranging from 1 hr to 7 d after the last treatment.

Locomotor activity

Automated locomotor activity chambers equipped with infrared photobeams (Opto-Varimex Minor; Columbus Instruments International Corporation, Columbus, OH) were used to quantify locomotor activity. Photobeam breaks were recorded each minute for 45 min for horizontal, vertical, and ambulatory movements. Mice were initially habituated to the locomotor activity chambers in three 45 min sessions occurring on consecutive days, with all mice receiving intraperitoneal vehicle injections before the session. After the third habituation session, treatments began, and effects on motor activity were assessed immediately and 24 hr after each injection (sessions 1 and 2, respectively) in 45 min test sessions with activity counts totaled in 3 min blocks across the session.

MPTP challenge

In a separate group of animals, 1 week after the 12th treatment, animals from each treatment group (i.e., saline, PQ, MB, or PQ + MB) were treated with either saline or 15 mg/kg MPTP ($n = 5$). Locomotor activity was assessed either immediately (see Fig. 3) or 24 hr after treatment (data not shown).

Permanent, progressive, or reversible effects of PQ + MB neurotoxicity

To address the issue of reversibility of the adverse effects of combined PQ and MB, 5-month-old mice were treated twice a week for 3 weeks with either saline, 10 mg/kg PQ, 30 mg/kg MB, or PQ + MB ($n = 6$ per group). Three months after the last injection, animals were killed, brains were removed, and tyrosine hydroxylase protein levels were determined using Western blot analysis (as described below).

Dopamine and metabolite analyses by HPLC

Neurotransmitter concentrations were measured 1 hr, 3 d, and 7 d after the 12th injection of either saline, PQ, MB, or PQ + MB. After rapid decapitation, striatal sections were dissected and placed in 0.1N perchloric acid. The tissues were sonicated and centrifuged for 8 min at 1000 × *g*. The supernatants were stored at −80°C until analyzed for the concentrations of DA, dihydroxyphenylacetic acid (DOPAC), and homovanillic acid by HPLC-EC. The pellets were digested in 1 ml of 0.5N NaOH for measurements of protein concentration using Bio-Rad (Hercules, CA) assay reagents. For HPLC-EC analysis, samples were loaded onto a Waters 717 plus autosampler (Waters Associates, Milford, MA), and the mobile phase was delivered at a constant rate of 1 ml/min by a Waters model 510 pump through a C18, 5 mm, 250 × 4.6 mm analytical column (Alltech, Deerfield, IL) placed in a column heater (35°C). The LC amperometric potential was set to 0.75 V with reference to an Ag–AgCl reference electrode, and the sensitivity of the detector was maintained at 2 nA. The mobile phase consisted of 0.1 M disodium phosphate, 0.1 M citrate, 0.15 mM EDTA, 1.4 mM octyl sodium sulfate, and 12% methanol. The signal from the detector was recorded, and the data were analyzed by the use of a Waters Millennium 2010 Chromatography Manager. The concentrations of the neurotransmitters were expressed as nanograms per milligram of protein. DA turnover was expressed as the ratio DOPAC/DA.

Immunohistochemistry for tyrosine hydroxylase and the dopamine transporter

Tissue preparation. After intracardial perfusion with heparinized saline followed by 4% paraformaldehyde (PFA), brains were harvested and post-fixed in 4% PFA for 2 hr and consequently cryoprotected in buffered sucroses to 30% over 2 d. Brains were cut into 40 μm sections and collected in cryoprotectant. Sections were stored at −20°C until used for immunohistochemistry (IHC).

Immunohistochemistry. Sections were washed with 0.1 M phosphate buffer (PB), blocked for nonspecific binding, and incubated with a 1° antibody (Ab) either to TH (Chemicon, Temecula, CA) or to the DA transporter (Chemicon) overnight at a dilution of 1:4000 in 0.1 M PB containing 0.3% Triton X-100 and 10% goat serum. Sections were subsequently washed and incubated with avidin–biotin solution using the Vectastain Elite kit (Vector Laboratories, Burlingame, CA) for 1 hr at room temperature. Sections were quenched for endogenous peroxidase by the use of hydrogen peroxide for 10 min. Sections were then developed in 3–3′-diaminobenzidine tetrachloride for 2–3 min. After several rinses, sections were mounted out of PB, dehydrated, and coverslipped.

Immunohistochemistry and astrocyte counts for glial fibrillary acidic protein

Tissue preparation was similar to that described for TH and the dopamine transporter (DAT). Sections were washed with 0.1 M PB, blocked, and incubated with anti-glial fibrillary acidic protein (anti-GFAP; Chemicon) at a dilution of 1:500 overnight at 4°C. Sections were then washed and incubated with an anti-rabbit Alexa 594 Ab (Molecular Probes, Eugene OR) at a dilution of 1:500 for 2 hr. Sections were then washed and mounted with mowiol (Polysciences, Warrington, PA). Fluorescent images were viewed using a TRITC filter, and reactive astrocytes were counted without the experimenter having knowledge of the treatment. The total number of clusters and total number of astrocytes were quantitated in the dorsal–medial striatum ($n = 6$ per treatment group). Clusters were defined as two or more reactive astrocytes adjacent to each other. Only reactive astrocytes expressing abundant GFAP were counted. Astrocytes that were close to white matter or were entangled with vasculature were omitted. The average numbers of astrocytes and clusters per section from each animal were used for statistical analysis.

Densitometric measurements of tyrosine hydroxylase and dopamine transporter immunoreactivity

Six serial striatal sections and four nucleus accumbens sections were used for densitometric measurements, with each treatment group represented by an $n = 6$. Densitometric measurements were performed using NIH Image software and with the experimenter blinded to the treatment groups. Images were acquired at the same magnification to capture the entire striatal area in a single field and converted to gray scale before the staining intensity was analyzed. Two different areas from each half of a section, encompassing 30% of the total area, were analyzed to ensure adequate representation of the entire area of interest. The density of the two regions was averaged to give a total mean density. Background was established by determining the density of an area that did not stain with anti-TH antibody.

Tyrosine hydroxylase protein levels by the use of Western blot analysis

After rapid decapitation, the striatum was removed and stored at −80°C until analysis. Protein concentrations were determined using the Bio-Rad assay reagents. Each treatment group had an $n = 8$. For each sample, three different concentrations of protein (5, 10, and 20 μg) were loaded onto separate lanes. The samples were subjected to electrophoresis, transferred to nitrocellulose, and immunoblotted with rabbit antiserum specific for TH. Detection was performed with the ECL kit using autoradiography. The autoradiograms were scanned, and the autoradiographic bands were quantitated using NIH Image software to calculate the density. Only density values that were within the linear range of the autoradiographic film were used (Osterhout et al., 1997). The density for each TH protein band was normalized to the amount of protein loaded onto the gel for that

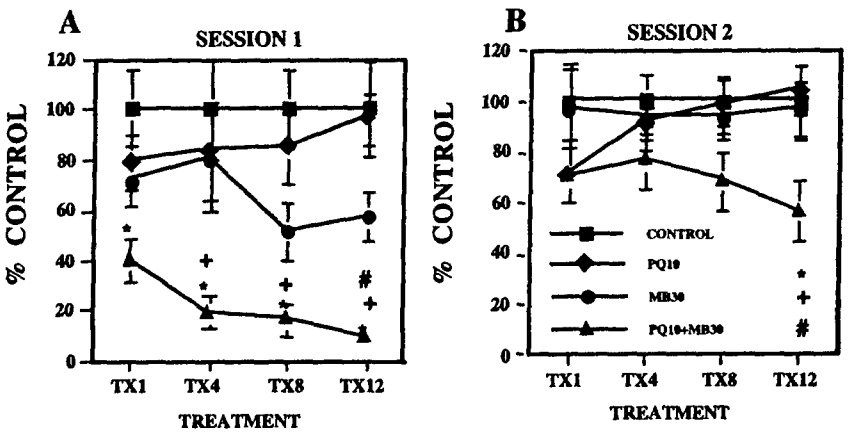


Figure 1. *A*, Total ambulatory locomotor activity. The group mean (± SE; $n = 10$; plotted as percent of control group values) was measured immediately after the 1st, 4th, 8th, and 12th injections (*TX1*, *TX4*, *TX8*, and *TX12*, respectively) of either saline (*CONTROL*), 10 mg/kg paraquat (*PQ10*), 30 mg/kg maneb (*MB30*), or their combination (*PQ10+MB30*). Bonferroni–Dunn *post hoc* tests for each treatment day indicate differences: *, from the control group; +, from PQ alone; #, from MB alone. *B*, Corresponding effects measured 24 hr after treatment.

particular sample and then divided by the density of the known amount of TH protein loaded onto that gel. TH protein values were expressed as micrograms of TH protein per milligram of protein loaded.

Tyrosine hydroxylase staining intensity in the substantia nigra and ventral tegmental area

Sections were stained as described under TH immunohistochemistry. Quantitative estimates of TH immunoreactivity were made in the bilateral substantia nigra pars compacta (SNc) and the ventral tegmental area (VTA). Intensity measurements were done with the experimenter blinded to the treatment and in random order to avoid any bias. A total of five representative sections per animal were used to determine TH density within the SNc, and two sections per animal were used for the VTA. Each treatment group was represented by an $n = 6$. The analysis, using the NIH Image software program, was done after encircling the SNc or VTA in each sampled section at a magnification of 40×, permitting representative visualization of each region. These parameters were constant across sampled sections. The average of the density for each section was added and divided by the total number of sections analyzed to give the mean TH density for each animal.

Cell counts in the substantia nigra and ventral tegmental area

The sampled sections from all of the animals ($n = 6$ per treatment group) were stained simultaneously by the use of the above described method for TH IHC. After being mounted on gelatin-coated slides, the sections were counterstained with cresyl violet to allow the visualization of TH-positive neuronal nucleoli. Three representative sections containing the SNc and the VTA were chosen. Quantitative counts of the total number of TH-immunoreactive cell bodies counterstained with cresyl violet (TH/cresyl violet-positive neurons) were made in the bilateral SNc and VTA. Counts were performed manually and with the experimenter blinded to the treatment received. The average number of neurons per section was added to provide a measure of the total number of TH/cresyl violet-positive neurons for each animal and then divided by the number of counted sections to give a mean of the number of labeled neurons per section.

Lung histopathology

Because PQ is known to target lung (Bus et al., 1976), representative sections of lung ($n = 6$) were prepared by formalin fixation, paraffin embedding, sectioning at 4 μm, and staining with hematoxylin and eosin. Sections were examined without knowledge of the treatment group for evidence of alterations in alveoli, respiratory ducts, bronchioles, and bronchi.

Statistical analysis

Overall effects of treatment on horizontal locomotor activity were first analyzed with repeated measure ANOVAs (RMANOVAs) using treatment as a between-groups factor and injections as a within-group factor. This was followed by individual one-way ANOVAs using treatment as a between-groups factor for each injection (see Fig. 1) and subsequent *post hoc* Bonferroni–Dunn tests to compare treatment groups. Bonferroni–Dunn tests control for numbers of comparisons and thus provide a conservative estimate of significance. To assess treatment-related changes within an activity session (see Fig. 2), RMANOVAs with treatment as a between-groups factor and time block as a within-group factor were used; significant main effects of treatment or interactions were followed by one-factor ANOVAs at each time point. Changes in DA, DOPAC, and turnover (see Fig. 4) were first evaluated using treatment and time point (1 hr, 3 d, and 7 d) as between-groups factors for ANOVA. This was followed by separate ANOVAs for each time point for each measure. Effects of all other end points were analyzed using one-factor ANOVA with treatment as the between-groups factor, followed by Bonferroni–Dunn tests in the event of significant main effects of treatment.

RESULTS

Body weight and lung pathology

No treatment-related changes in body weights were observed at any point in the experiments, either (1) when body weights before each motor activity session were compared across the entire experiment or (2) when body weights from the final habituation session before treatment were compared with (a) body weights on the 12 injection days, (b) body weights on the day after each injection, or (c) body weights on the last day of the experiment. Lungs were graded for signs of alveolitis, bronchiolitis, bronchitis, lymphoid aggregation, bronchiectasis, and fibrosis and found to be histologically normal.

Locomotor activity

Total ambulatory activity counts determined immediately after injections (Fig. 1*A*, session 1) were reduced only by PQ + MB [main effect of treatment, $F_{(3,36)} = 4.11$ ($p = 0.0132$); $F = 4.26$ ($p = 0.0113$); $F = 8.62$ ($p = 0.0002$); and $F = 12.39$ ($p < 0.0001$), for treatments 1, 4, 8, and 12, respectively]. By the 12th injection, the motor activity levels of the combined PQ + MB group were significantly lower than were those of all other groups, having decreased to 9% of control. Although an emerging reduction in activity in response to MB alone is suggested after treatments 8 and 12, these effects were not statistically different from control at either time point.

An emergent failure in the recovery of motor activity measured 24 hr after injection was also observed in the combined PQ + MB group (Fig. 1*B*). Although recovery was complete after the 1st, 4th, and 8th injections, values of the PQ + MB group evidenced only partial recovery by the 12th injection [main effect of treatment, $F_{(3,36)} = 3.52$ ($p = 0.0245$)] and were significantly lower than those of the PQ-only group and marginally lower than those of the control and MB-only groups.

Potentiated effects of combined PQ + MB were especially evident across the course of behavioral sessions. As shown after the 4th and 12th injections (Fig. 2*A,C*), PQ + MB significantly reduced motor activity relative to that of the control and PQ-alone groups (not indicated on figure) at virtually every time point and relative to that of the MB-only group during the final half of the session at both time points [interactions of time by treatment, $F_{(42,504)} = 2.23$ ($p < 0.0001$); and $F = 3.14$ ($p < 0.0001$), respectively]. Parallel changes were noted in both horizontal and vertical motor activity (data not shown). A suggestion of lower motor activity levels during the first half of the session after the 4th MB-alone injections was enhanced with continuing treatment, producing significant reductions in motor activity relative to that in controls with recovery to control levels during the final half of the session after the 12th injection.

Within 24 hr after treatment 4 (Fig. 2*B*), all groups exhibited full recovery. By the 12th injection, however, the combined PQ + MB group was no longer exhibiting full recovery of motor activity within 24 hr [Fig. 2*D*, main effect of treatment, $F_{(3,14)} = 3.52$ ($p = 0.0245$)], with levels now remaining at only ~50% of those of controls.

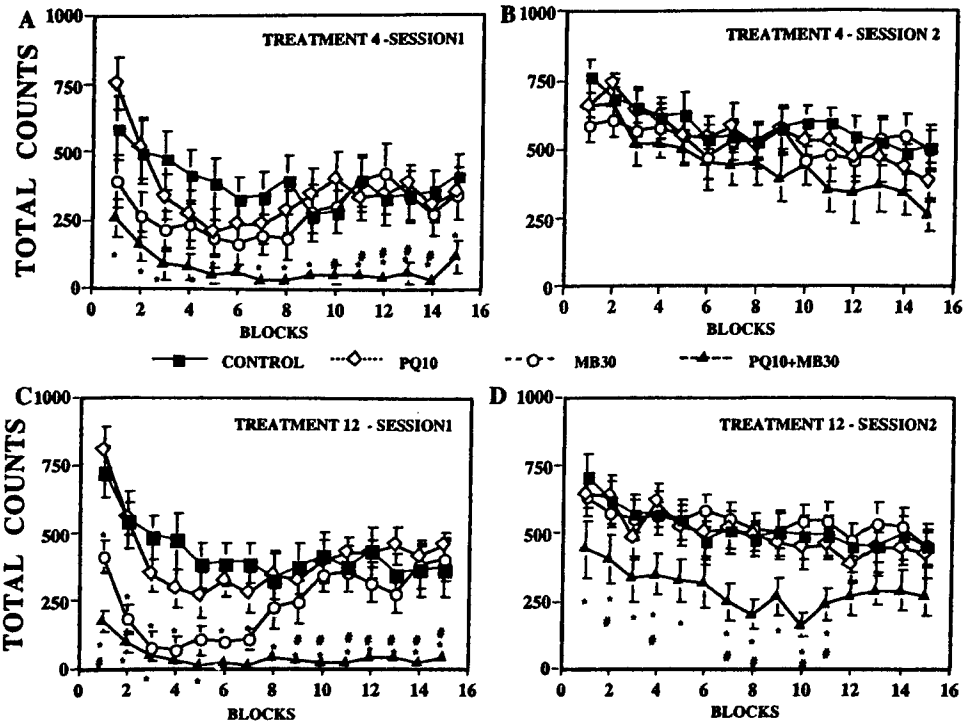


Figure 2. Ambulatory locomotor activity across a behavioral session. The group mean (± SE) was measured in 3 min blocks across a 45 min behavioral session immediately after the 4th and 12th treatment (*A, C,* respectively) or 24 hr after treatment (*B, D,* respectively) with vehicle (*CONTROL*), 10 mg/kg PQ (*PQ10*), 30 mg/kg MB (*MB30*), or their combination (*PQ10+MB30*). Significant differences are as follows: *, from the control group; #, from maneb alone. Significant differences of the combined PQ + MB group from PQ alone are not shown. Sample sizes are as noted in the Figure 1 legend.

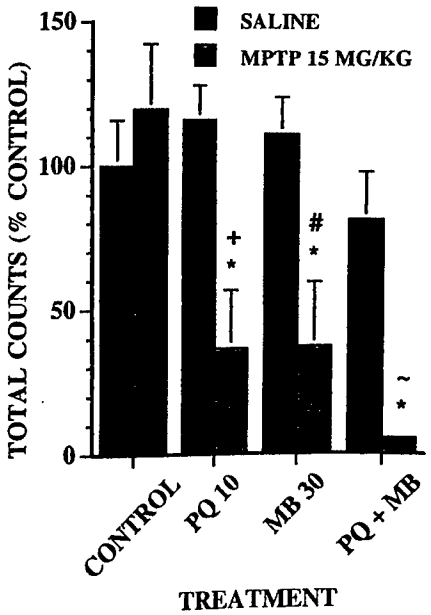


Figure 3. Effect of an acute dose of MPTP on locomotor activity. The group mean (± SE) of total ambulatory locomotor activity counts in 45 min sessions was measured immediately after intraperitoneal injections of saline (n = 5) or 15 mg/kg MPTP (n = 5) after 12 treatments with vehicle (*CONTROL*), 10 mg/kg PQ (*PQ 10*), 30 mg/kg MB (*MB 30*), or their combination (*PQ + MB*). Data are plotted as the percent of the saline control group values. Bonferroni–Dunn *post hoc* tests for each treatment day indicate differences: *, from the control saline group; +, from the PQ saline group; #, from the MB saline group; ~, from the PQ+MB saline group.

MPTP challenge

Total locomotor activity counts after saline or a challenge dose of 15 mg/kg MPTP (Fig. 3) were modulated by treatment [main effect of MPTP, $F_{(3,16)}$ = 8.11 (p = 0.0016); treatment by MPTP interaction, $F_{(3,31)}$ = 4.74 (p = 0.0078)]. This dose of MPTP did not affect motor activity levels of controls. MPTP significantly reduced activity by 63–64% relative to control in both the PQ-alone and MB-alone groups. Although the largest decline was found in the PQ + MB group (95%), it was not significantly greater than that detected in the PQ-alone and MB-alone groups.

Reversibility of PQ + MB neurotoxicity

Three months after the last treatment of PQ or MB alone, striatal TH protein levels were comparable with those of saline-treated animals. However, mice treated with PQ + MB showed a 39% decrease in striatal TH protein levels relative to that in saline controls [main effect of treatment, $F_{(3,20)}$ = 5.555 (p = 0.0061)]. TH protein levels of the PQ + MB group were significantly lower than those in control, PQ-alone, and MB-alone groups in *post hoc* analysis (p = 0.004, 0.001, and 0.02, respectively).

Striatal dopamine, DOPAC, and turnover

Changes in levels of DA, DOPAC, and DA turnover, measured at 1 hr, 3 d, and 7 d after the 12th injection (Fig. 4), differed in relation to time point [$F_{(6,70)}$ = 3.20 (p = 0.0078); F = 4.66 (p = 0.0005); and F = 3.83 (p = 0.0023), for DA, DOPAC, and DA turnover, respectively]. Subsequent one-factor ANOVAs at each time point for each measure revealed that levels of DA were significantly elevated by 20–30% 1 hr after injection in the PQ-alone, MB-alone, and PQ + MB groups. Values in the PQ + MB group, although somewhat higher, differed only marginally from the PQ-alone and MB-alone groups (p = 0.09 and 0.10, respectively). Corresponding increases in DOPAC at 1 hr were produced by MB alone and by PQ + MB, with PQ + MB group values (>40% control) significantly higher than the corresponding control and PQ-alone group values. DA turnover was increased at 1 hr only in the PQ + MB group, at least relative to PQ alone.

Three days after the 12th treatment, residual increases in DA levels differed only marginally from control (p = 0.06), because of both the MB-alone (p = 0.014) and the PQ + MB groups (p = 0.032). DOPAC had recovered to control levels. DA turnover was significantly suppressed at this time point only in the PQ + MB group [main effect of treatment, $F_{(3,27)}$ = 5.40 (p = 0.0048)], compared with both the control and PQ-only groups (p = 0.0007 and 0.005, respectively).

By day 7, a trend toward reductions of DA, DOPAC, and turnover was suggested and was marginally significant for DOPAC (p = 0.085) because of differences between controls and the PQ + MB group (100 vs 73%; p = 0.014).

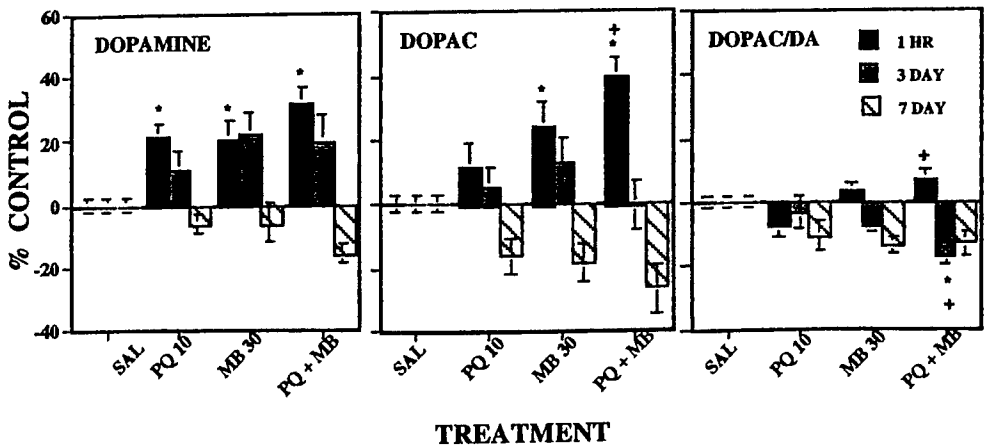


Figure 4. Striatal levels of DA (*left*), DOPAC (*middle*), and DA turnover (DOPAC/DA; *right*). The changes in group mean (± SE) from control levels of DA, DOPAC, and DA turnover were assessed either 1 hr (n = 10), 3 d (n = 10), or 7 d (n = 5) after the 12th intraperitoneal injection of vehicle (*SAL*), 10 mg/kg paraquat (*PQ 10*), 30 mg/kg maneb (*MB 30*), or their combination (*PQ + MB*). Data are plotted as a percent of the control group value for the corresponding time point. Bonferroni–Dunn *post hoc* analysis indicates significant differences: *, from the control group; +, from PQ alone; #, from MB alone.

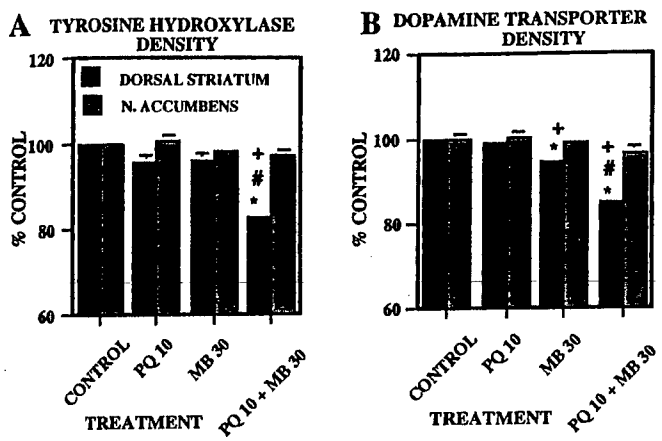


Figure 5. Striatal immunohistochemistry of TH and DAT. Group mean (± SE) levels of TH (*A*) and DAT (*B*) immunoreactivity (expressed as percent of control group values) in dorsal striatum and nucleus (*N.*) accumbens were measured 5 d after 12 treatments with vehicle (*CONTROL*; n = 6), 10 mg/kg paraquat (*PQ 10*; n = 6), 30 mg/kg maneb (*MB 30*; n = 6), or their combination (*PQ 10 + MB 30*; n = 6). Bonferroni–Dunn *post hoc* analysis indicates significant differences: *, from the control group; +, from PQ alone; #, from MB alone.

Striatal TH and dopamine transporter immunoreactivity

Levels of TH and DAT immunoreactivity determined 5 d after the 12th injection (Fig. 5) were reduced 15–17% by PQ + MB relative to the levels in all other groups in the dorsal striatum [$F_{(3,20)}$ = 24.7 ($p < 0.0001$); and F = 54.1 ($p < 0.0001$), respectively; Bonferroni–Dunn tests, all p values < 0.0001], whereas no effects of treatment were detected in the nucleus accumbens (p = 0.18 and 0.22, respectively). Striatal DAT immunoreactivity was also reduced ~6% by MB alone relative to the control and PQ-only groups (p = 0.0005 and 0.0032, respectively).

TH protein levels

Striatal TH protein levels (Fig. 6*B*) were unaffected by treatment 1 hr after the 12th injection. However, by the 5 d time point, PQ + MB treatment had reduced TH by ~35% [main effect of treatment, $F_{(3,16)}$ = 4.46 (p = 0.0185)]. These values were significantly lower than were those of both the control and PQ-only groups (p = 0.005 and 0.007, respectively), as evident in the corresponding bands from Western blots (Fig. 6*A*), particularly at the higher protein-loading levels.

TH immunoreactivity and cell counts in the substantia nigra and ventral tegmental area

Levels of TH intensity and cell counts 5 d after the 12th injection (Fig. 7) were reduced only by PQ + MB (~32%) relative to all other groups in the substantia nigra [main effect of treatment, $F_{(3,20)}$ = 32.23 ($p < 0.0001$); Bonferroni–Dunn tests, all p values < 0.0001], whereas there were no effects in the ventral tegmental area [$F_{(3,20)}$ = 0.21 (p = 0.89)]. Cell counts were reduced only by PQ + MB and only in the substantia nigra [main effect of treatment, $F_{(3,20)}$ = 3.39 (p = 0.038); p = 0.89 for ventral tegmental area], dropping by 22% relative to the control group.

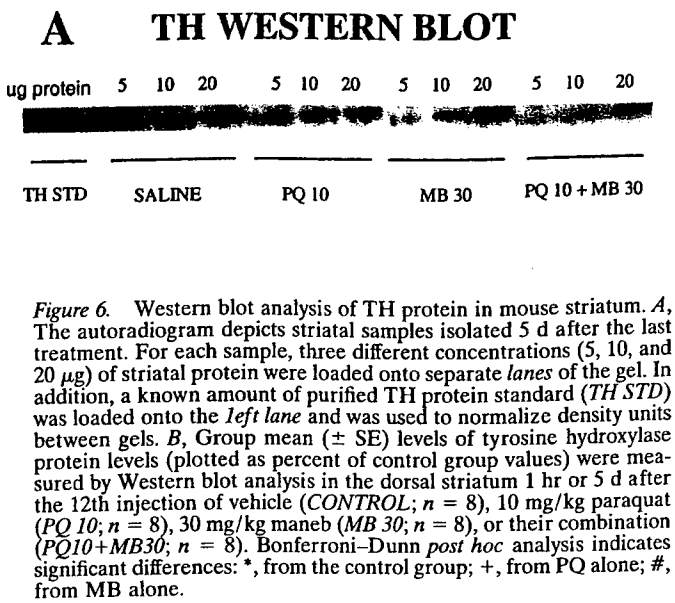
Glial fibrillary acidic protein immunoreactivity

GFAP IHC stains both resting and reactive astrocytes in the mouse brain. Resting astrocytes are easily identified in white matter tracts and several nuclei, but not in the striatum. Reactive astrocytes are readily seen in the striatum of combined PQ + MB animals and rarely seen in saline animals. Reactive astrocytes in the striatum tended to occur in clusters of 2–20. Combined treatment with PQ + MB resulted in gliosis in the dorsal (Fig. 8) but not the ventral striatum, as indicated by counts of either the total number of reactive astrocytes or total number of clusters of reactive astrocytes (Table 1). The total number of reactive astrocytes was nonsignificantly increased to 169 and 161% in response to PQ alone and MB alone, respectively, but rose to 373% in the combined PQ + MB group [main effect of treatment, $F_{(3,19)}$ = 4.63 (p = 0.0136)] compared with controls. When counted as total numbers of "clusters," the combined PQ + MB group increased the number of clusters relative to those in control, PQ only, and MB only to levels of 275% of control [main effect of treatment, $F_{(3,20)}$ = 7.14 (p = 0.0019)].

DISCUSSION

This study examined the premise that protracted exposure to subclinical or minimally effective doses of PQ and MB could, when combined, produce additive or even synergistic effects by simultaneously impacting multiple target sites of the DA system and precluding homeostatic reregulation. Indeed, combined PQ + MB produced synergistic effects targeting the nigrostriatal DA system that were progressive in nature and consistent with neurotoxicity, validating the model suggested by our previous findings (Thiruchelvam et al., 2000). Sustained reductions in locomotor activity immediately after injection and failure to evidence full recovery 24 hr later were observed only in the PQ + MB group. PQ + MB, but not either alone, reduced striatal but not nucleus accumbens TH and DAT immunoreactivity, striatal TH protein levels, and TH immunoreactivity and cell counts in the substantia nigra but not in the VTA and produced reactive gliosis in the dorsal striatum but not in the nucleus accumbens. Furthermore, a dose of MPTP (15 mg/kg) without effects in control mice markedly decreased locomotor activity in groups receiving PQ, MB, or PQ + MB. Thus, previous exposure to PQ and/or MB enhances nigrostriatal system vulnerability to the adverse effects of the selective nigrostriatal neurotoxin MPTP, further confirming adverse effects of PQ and MB on this system.

These findings also indicate progressive neurotoxicity with continuing exposure. The more protracted exposures used here (12 injections) increased the magnitude of adverse effects relative to those seen after 4 injections (Thiruchelvam et al., 2000). TH



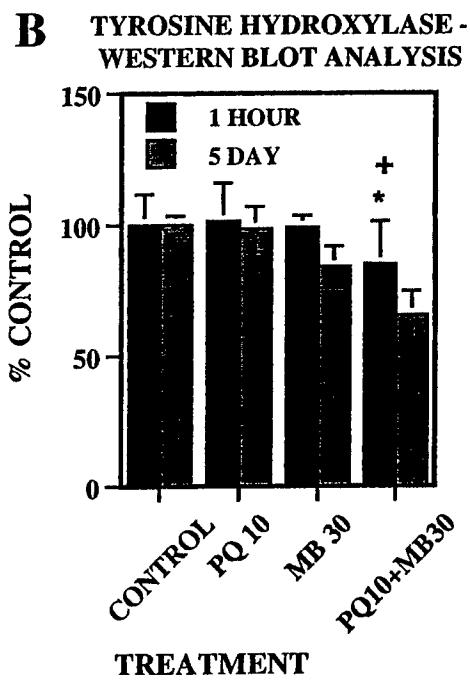


Figure 6. Western blot analysis of TH protein in mouse striatum. *A*, The autoradiogram depicts striatal samples isolated 5 d after the last treatment. For each sample, three different concentrations (5, 10, and 20 μg) of striatal protein were loaded onto separate *lanes* of the gel. In addition, a known amount of purified TH protein standard (*TH STD*) was loaded onto the *left lane* and was used to normalize density units between gels. *B*, Group mean (± SE) levels of tyrosine hydroxylase protein levels (plotted as percent of control group values) were measured by Western blot analysis in the dorsal striatum 1 hr or 5 d after the 12th injection of vehicle (*CONTROL*; $n = 8$), 10 mg/kg paraquat (*PQ 10*; $n = 8$), 30 mg/kg maneb (*MB 30*; $n = 8$), or their combination (*PQ10+MB30*; $n = 8$). Bonferroni–Dunn *post hoc* analysis indicates significant differences: *, from the control group; +, from PQ alone; #, from MB alone.

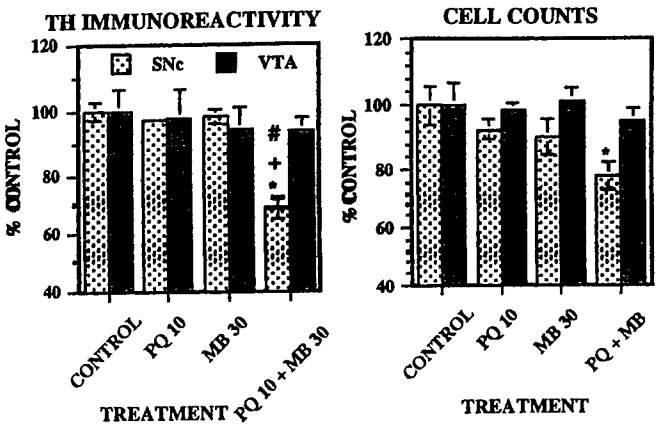


Figure 7. TH immunoreactivity and neuronal cell counts in the substantia nigra and ventral tegmental area. Group mean (± SE) levels of TH immunoreactivity (*left*) and total cell counts (*right*) were measured 5 d after the 12th injection of vehicle (*CONTROL*; $n = 6$), 10 mg/kg paraquat (*PQ 10*; $n = 6$), 30 mg/kg maneb (*MB 30*; $n = 6$), or their combination (*PQ 10 + MB 30*; $n = 6$). Bonferroni–Dunn *post hoc* analysis indicates significant differences: *, from the control group; +, from PQ alone; #, from MB alone.

density declined 10% after 4 injections and 17% after 12 PQ + MB injections. Although motor activity levels had returned to control values within 24 hr after 4 injections, a failure to recover fully had emerged by treatment 12. Furthermore, increases in DA and DOPAC tended to remain elevated even 3 d after 12 injections, whereas corresponding values after 4 injections were below control group values. Moreover, these effects appear to be irreversible; 5-month-old mice that received six injections of PQ + MB showed 39% reductions in striatal TH protein levels when measured 3 months after treatment ended. Reversibility, or the extent of recovery, may, however, depend on the age at which exposures are imposed, and ongoing studies will assist in addressing this issue.

The collective findings suggest that PQ + MB may be an environmental analog of the methamphetamine model of PD. Repeated methamphetamine produces surges of DA that ultimately reduce DA and metabolites and DA transporter and TH activity and produces reactive gliosis in the striatum and nigra (Kogan et al., 1976; Sonsalla et al., 1989; O'Dell et al., 1991; Bowyer et al., 1998; Escubedo et al., 1998). In one study, cell loss in the substantia nigra was reported (Sonsalla et al., 1996). Indeed, DA infusions into striatum produce neuronal loss and reactive gliosis (Filloux and Townsend, 1993; Hastings et al., 1996). PQ + MB injections also engendered surges of DA and metabolites and of DA turnover ultimately accompanied by reductions of TH and DAT density and of TH protein in striatum, consistent with terminal dysfunction or more likely a loss of terminals. Either of these outcomes could further elevate DA and metabolite levels. Although the striatum contains serotonergic and cholinergic neurons, the decreases of TH and DAT immunoreactivity in the striatum and of TH immunoreactivity in the substantia nigra argue that GFAP elevations reflect damage to DA components. DA might exert neurotoxic effects via enzymatic metabolism forming H_2O_2 that can be broken down to free radical species in the presence of metals (Cohen, 1984; Spina and Cohen, 1989; Olanow and Tatton, 1999) or via auto-oxidation forming various reactive compounds (Halliwell and Gutteridge, 1984). Without direct evidence, however, the possibility that PQ + MB exposure damages other striatal neurochemical systems cannot yet be excluded.

The surges in DA observed 1 hr to 3 d after PQ + MB probably underlie the corresponding suppression in motor activity. Indirect DA agonists such as cocaine and amphetamine increase activity at low doses but at higher doses decrease activity, probably reflecting their ability to provoke stereotyped behaviors (Ansah et al., 1993; Rosenzewig-Lipson et al., 1997). Although both PQ and MB alone also increased DA 1 hr after the 12th injection, these effects had a later onset in the course of treatment; they were not evident after four injections as were increases in DA in response to PQ + MB (Thiruchelvam et al., 2000) and thus may not have yielded sufficient DA surges to evoke corresponding changes in motor activity or nigrostriatal system neurotoxicity. Interestingly, only PQ + MB also resulted in an increase in DA turnover after 12 injections, indicating a more substantial involvement of DA system changes with the combined exposure.

The basis for selective vulnerability of the nigrostriatal system to PQ + MB is not yet clear. PQ + MB reduced TH and DAT striatal immunoreactivity in dorsal striatum, but not in nucleus accumbens, and reduced TH immunoreactivity and cell counts in substantia nigra pars compacta, but not in VTA. Reactive gliosis was observed in dorsal striatum but not nucleus accumbens. In PD, pigmented melanized DA neurons are targeted, and there is a greater number of pigmented neurons in substantia nigra (>80%) than in VTA (50%) (Hirsch et al., 1989; Hirsch and Faucheux, 1998). PQ, like MPTP, has a propensity to accumulate in melanin-containing neurons. However little neuromelanin exists in mouse

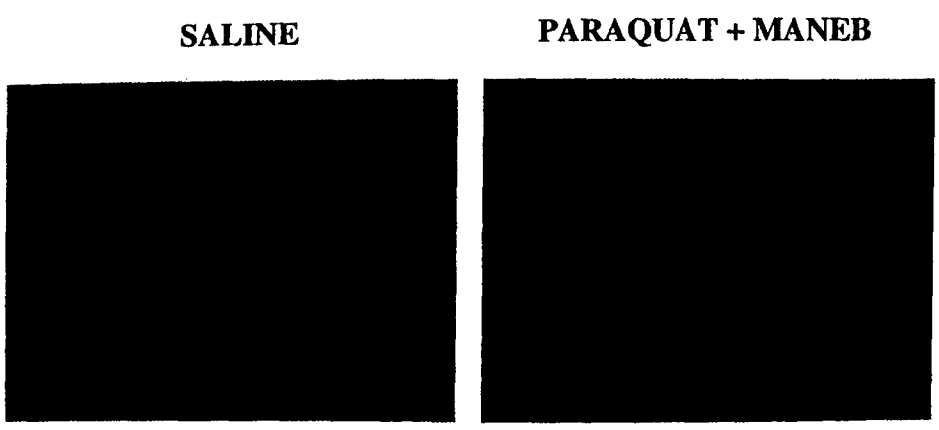


Figure 8. GFAP immunoreactivity in dorsal striatum. Representative photomicrographs depicting GFAP immunoreactivity 5 d after the 12th injection of vehicle (*A*) or the combination of 10 mg/kg paraquat and 30 mg/kg maneb (*B*) in dorsal striatum. The combined treatment shows distinguishable clusters of reactive glia that were not observed after corresponding treatment with vehicle or PQ or MB alone.

Table 1. GFAP immunoreactivity in dorsal striatum

Treatment	Total number of astrocytes	% of control number astrocytes	Total number of clusters	% of control number of clusters
Saline	11 ± 2.2	100 ± 18.5	3 ± 0.8	100 ± 45.1
Paraquat (10 mg/kg)	20 ± 5.4	169 ± 46	4 ± 0.88	130 ± 26.5
Maneb (30 mg/kg)	19 ± 4.3	161 ± 36.5	5 ± 0.62	140 ± 18.4
Paraquat + maneb	44 ± 11*	373 ± 98.2*	9 ± 1.4*†#	275 ± 42*†#

Numbers of reactive astrocytes 1 ($n = 6$), PQ (10 mg/kg; $n = 6$), MB (30 mg/kg; $n = 6$), and PQ + MB ($n = 5$) groups. Bonferroni–Dunn *post hoc* analysis indicates significant differences from either control paraquat alone or maneb alone.

Significantly different: *, from control; †, from paraquat alone; #, from maneb alone.

brain (Barden and Levine, 1983), suggesting that other sites must be targeted. The cause(s) for the synergistic effects of PQ + MB cannot be discerned from the current study. One possibility involves alterations in kinetics and consequent brain uptake. Interestingly, diethyldithiocarbamate can increase MPP^+ levels in brain after MPTP administration (Irwin et al., 1987). It is conceivable that MB, an ethylenebisdithiocarbamate, increases PQ uptake into brain, because PQ is structurally similar to MPP^+. PQ injected directly into nigra does produce neurotoxicity and nigrostriatal system degeneration (Liou et al., 1996).

PQ + MB could act via several different mechanisms to alter DA function. PQ, by redox cycling, can generate oxygen free radicals (Clejan and Cederbaum, 1989) that exert cytotoxic effects by disrupting mitochondrial complex 1 activity (Fukushima et al., 1993; Tawara et al., 1996). MB can alter vesicular glutamate uptake (Fukushima et al., 1993; Tawara et al., 1996; Vaccari et al., 1998, 1999) and storage and release of striatal DA (Vaccari et al., 1996). The ethylenebisdithiocarbamate anion and not the manganese moiety of MB appears to be the active component (Soleo et al., 1996) and possesses chelating properties. Thus, it could chelate metals such as iron, which have been shown to accumulate in brains of PD patients (Lan and Jiang, 1997). These possibilities are consistent with the assertion that targeting multiple sites of the nigrostriatal DA system may impede homeostatic reregulation, resulting in toxicity.

Doses of PQ and MB used here were not associated with overt toxicity, body weight changes, or lung pathology. The MB dose is <1% and the PQ dose is possibly 10–33% of reported LD_{50} values (Bus et al., 1976; Berg, 1977; Drew and Gram, 1979; Yamamoto, 1993). Information on actual human exposure levels to these compounds, which may occur via dermal, inhalation, and/or oral exposures, is not readily available. That PQ + MB exposures could occur concurrently is suggested by overlap in geographical use and the fact that maximal residue levels for both are established for food products. Maneb and mancozeb occur as residues on foods (Newsome, 1976; Patsakos et al., 1992; Yamamoto, 1993). Increases in health complaints related to drifts of paraquat sprays have been reported (Ames et al., 1993). Paraquat is used on cotton, and cotton waste can be used as a food supplement for beef cattle. Although presumably at much lower levels than used here, doses in the current study were imposed over only a 6 week period, unlike the much more protracted exposures that might be expected in human populations. It also seems unlikely that the effects observed here would be restricted to the combination of PQ + MB. MB is only one member of a larger family of dithiocarbamates used in geographical overlap with PQ. Numerous other agrichemicals, including organophosphates, share these patterns of geographical use and are also found as residues in foods. Thus, there is clearly a basis to suppose that supramixtures of agricultural chemical exposures may be likely. Our findings, therefore, may represent only a preliminary assessment of the role of environmental agrichemicals as risk factors for PD.

Evidence from this study also suggests that prolonged exposures to PQ and MB alone may result in progressive effects. As exposure progressed, systemic MB reduced locomotor activity, enhanced DA levels and susceptibility to MPTP, and decreased DA transporter density. Although effects of systemic PQ exposures have been equivocal (Ames et al., 1993; Widdowson et al., 1996a,b), this study appears to be the first to report that a behaviorally silent dose of PQ can potentiate MPTP's effects on motor activity and also ultimately increase DA levels. Effects of PQ alone may be enhanced by blood–brain barrier disruption (Brooks et al., 1999), raising questions about the extent to which such combined exposures either early in development or during advanced age may enhance brain uptake of these agrichemicals. Interestingly, mice exposed to 0.36 mg/kg PQ at postnatal day 10–11 show permanent hypoactivity and attenuated levels of striatal DA (Fredriksson et al., 1993). Uptake of PQ into brain is age dependent, with higher concentrations of PQ detected in very young (2-weeks-old) and old (24-months-old) animals (Corasaniti et al., 1991; Fredriksson et al., 1993; Widdowson et al., 1996a).

Risk assessment guidelines for human exposure to compounds such as those used here are typically based on levels producing no effect derived from exposures to single agents. Our findings show, however, that such compounds, while having no or marginal effects when administered individually, can produce synergistic effects when given in combination. This obviously implies that the current derivation of risk assessment guidelines needs to be reevaluated. Finally, it is unlikely that such exposures per se produce PD. A more probable etiological basis involves gene–environment interactions, in which such exposures would act in conjunction with a

susceptible genetic predisposition. Thus future studies imposing such exposures on genetic backgrounds of potential vulnerability could further advance the understanding of PD.

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IN VITRO AUGMENTATION OF DOPAMINE UPTAKE BY FUNGICIDES.

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Fungicides are implicated as a risk factor for Parkinson's disease (PD) based on epidemiological data in man, animal models, and *in vitro* biochemical studies. Several members of the dithiocarbamate (DTC) class of fungicides are known to increase the toxicity of both MPTP and paraquat in mice by a yet unknown mechanism. Since the dopamine transporter (DAT) has been proposed to be a gateway for dopaminergic neurotoxicants, we sought to determine if these DTCs have a common mechanism of toxicity acting via this transporter. We measured dopamine (DA) uptake in synaptosomes prepared from striata of C57BL/6 mice and the effect of different concentrations of compounds from two different classes of DTCs, diethyldithiocarbamates (DEDCs) and ethylene bis-dithiocarbamates (EBDCs). We calculated the K_m and V_{max} of the DAT for DA in the presence and absence of a single concentration of one type of DTC. There were concentration dependent increases in DA uptake for different compounds from the two classes of DTCs. Significant effects were seen at concentrations as low as 500 nM (15 to 53% increase), with maximal effects at 5-10 μM (27 to 70% increase). These effects were due to an increase in the V_{max} of the DAT for DA and not related to a change in K_m. These results suggest a mechanism by which the DTCs alter the DAT to influence the uptake of DA and perhaps other compounds traversing the DAT. DTCs may contribute to PD by augmenting the uptake of DAT-linked neurotoxicants (MPTP) or by increasing the concentration of DA in terminals, both mechanisms being potentially deleterious.

Physiologic and Pathologic Effects of Wild-Type and Mutated Human α-Synuclein in Transgenic Mice

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Abstract

Human α-synuclein (hα-SYN) is implicated in the Parkinson's disease phenotype (PDP) based on a variety of studies in man, animal models, and *in vitro* studies. The normal function of hα-SYN and the mechanism by which it contributes to the PDP remains unclear. We created transgenic mice expressing either wild-type (hwα-SYN) or a doubly-mutated (hm^{2}α-SYN) form of hα-SYN. These mice expressed hα-SYN in cell bodies, axons, and terminals of the nigrostriatal system. The expression of hα-SYN in nigrostriatal terminals produced physiological effects in both constructs resulting in increased density of the dopamine transporter (DAT) and enhanced toxicity to the neurotoxin MPTP. Expression of hm^{2}α-SYN reduced locomotor responses to repeated doses of amphetamine and blocked the development of sensitization. Adult hwα-SYN-5 transgenic mice had unremarkable dopaminergic axons and terminals, normal age-related measures on two motor coordination screens and normal age-related measures of dopamine (DA) and its metabolites. Adult hm^{2}α-SYN-39 transgenic mice had abnormal axons and terminals, age-related impairments in motor coordination and age-related reductions in DA and its metabolites. Expression of hm^{2}α-SYN adversely affects the integrity of dopaminergic terminals and leads to age-related declines in motor coordination and dopaminergic markers.

Key Words: α-synuclein, transgenic mice, Parkinson's disease, nigrostriatal system, substantia nigra, dopamine, MPTP

Introduction

Parkinson's disease (PD) is characterized by neuronal loss, inclusions, and gliosis in the substantia nigra (SN) resulting in dopamine (DA) deficiency in projection regions and clinical symptoms when the loss is extensive (17). The Parkinson's disease phenotype (PDP) results from different mechanisms including neurotoxin exposure or mutations including those in the α-synuclein (α-SYN) gene (20, 35). The causes of the PDP are poorly understood, but reflect risks associated with genetic background, environmental exposures, and aging (4). The human (hα-SYN) gene is implicated based on two known mutations (A53T and A30P) associated with autosomal dominant forms of the PDP, the presence of α-SYN in aggregates (2, 42), and possibly the presence of a polymorphism in the 5′ untranslated region of the hα-SYN gene associated with increased risk (18, 21). The function of α-SYN is unclear, but a role in presynaptic function exists (1, 6). Overexpression of α-SYN in cell culture (33), transgenic mice (25), and transgenic *Drosophila* (10) suggest adverse effects and the generation of aggregates.

The first reported transgenic mice expressing wild-type hα-SYN gene demonstrated inclusions and motor dysfunction with reduced tyrosine hydroxylase (TH) at an older age (25). Other transgenic models identified abnormalities in neurons and processes (23, 44), but did not identify differences between wild-type and mutated hα-SYN. Other transgenic mice have failed to demonstrate a phenotype (26). We created transgenic mice using the 9 Kb rat TH promoter to express either wild-type or a doubly-mutated form of hα-SYN in dopaminergic neurons. In these mice hα-SYN is functionally active in DA terminals and acts in part through the dopamine transporter (DAT). Expression of hm^{2}α-SYN leads to altered DA terminals with reduced response to repeated

administration of amphetamine and DA terminal failure with aging resulting in progressive motor impairment. We propose mutations in hα-SYN that result in the PDP may do so either by a gain of a toxic action or by interfering with the activity of hwα-SYN acting in DA terminals and mediated in part through the DAT.

Experimental Procedures

Generation and screening of transgenic mice. A clone (ID #48811) containing the full length wild-type hα-SYN cDNA was obtained from the I.M.A.G.E. consortium (Research Genetics Inc., Huntsville, AL). A 469 bp fragment of the coding sequence was obtained using the PCR with forward (hSYNU1, 5′-caggtaccgacagttgtggtgtaaaggaat) and reverse (hSYNL1, 5′-gatagctataaggcttcaggttcgtagtct) primers and subcloned into pGEMT (Promega Corp, Madison, WI). This vector was subjected to two rounds of *in vitro* mutagenesis (QuikChange™Site-Directed Mutagenesis kit, Stratagene Cloning Systems, La Jolla, CA.) with mutagenesis primers hSYNmut1U (ggagtggtgcatggtgtgacaacagtggctgagaagacc) and hSYNmut1L (ggtcttctcagccactgttgtcacaccatgcaccactcc) to introduce the G209A mutation and primers hSYNmut2U (cagggtgtggcagaagcaccaggaaagacaaaag) and hSYNmut2L (cttttgtctttcctggtgcttctgccacaccctg) to introduce the G88C mutation. All constructs were sequenced. The hwα-SYN and hm^{2}α-SYN genes were subcloned into pUTHTV. pUTHTV is a cloning vector constructed from pBluescript II KS (pBSKS, Stratagene Cloning Systems, La Jolla, CA.) by first introducing 9 additional rare restriction endonuclease sites in the multiple cloning site to create pEKR.3. The 9 kb rat tyrosine hydroxylase promoter (29, 41), a splice donor/intron/splice acceptor (15), and a polyA site from the human growth hormone gene (40) were subcloned from 5′ to 3′ into pEKR.3 to create pUTHTV. Subcloning of hwα-SYN into pUTHTV resulted in pUTHTV/hwα-SYN and subcloning of hm^{2}α-SYN into pUTHTV resulted in pUTHTV/hm^{2}α-SYN. The transgenes were removed by a Pac I digest, purified, and injected into oocytes obtained from C57/BL6 females. Founders and offspring were screened using the original PCR primers. The two constructs can be identified by restriction digests using Mwo I following the PCR.

Mice. Mice were group or individually housed in microisolation cages, given free access to food and water, kept on a 12 hr. light/dark cycle, and cared for as approved by the University of Rochester Committee on Animal Resources. Mice were sacrificed by rapid cervical dislocation followed by rapid dissection of regions or whole brain removal followed by freezing and storage at -20° C. Other mice were anesthetized with Nembutal and perfused transcardially 4% fresh paraformaldehyde (PFA). Perfused brains were post-fixed for an additional 2 hours in 4% PFA, processed through a graded series of sucrose and stored at 4°C.

Unless otherwise stated, young mice (age 2-3 months) were used for all characterization studies. We used middle-aged (7-9 months) and old-aged (13-23 months) mice for selected behavioral and neurochemical studies.

Southern Analysis. Tail genomic DNA was digested using Nco I and resulted in a fragment of 443 bp that was identified using a ^{32}P-labeled probe to the PCR product made using primers described earlier (hSYNU1 and hSYNL1).

Reverse transcriptase polymerase chain reaction (RT-PCR). RT-PCR was performed on mRNA from brain, eyes, and adrenal gland. mRNA was obtained using the MicroPoly(A)Pure™ kit (Ambion, Austin, TX). mRNA was reverse transcribed using the RETROScript™ kit (Ambion, Austin, TX) with 1-2 µl of product used with PCR primers (SDU3, ttctggagccggaggg and 3'UTRL1, cgatgcgcaggaatgtct). These primers cross the SD/SA region and will detect a product of size 1136 bp in genomic DNA and 846 bp in a mRNA/cDNA product that has been appropriately spliced.

Real-time quantitative reverse transcriptase polymerase chain reaction (QRT-PCR). QRT-PCR was performed after obtaining mRNA, which reverse transcribed as described earlier and 1-2 µl of product were used along with PCR primers (3'UTRFwd, ggcagctagaagccacagct and 3'UTRRev,

tggacaaggtcgagacattcc) directed to the 3′ UTR region of the transgene and a FAM labeled probe (FAM-cggcactgcacgatgcgca-TAMRA).

***In situ* hybridization histochemistry (ISHH).** ISHH was performed using a 48 mer DNA oligonucleotide probe (aatgtctcgaccttgtccatgtccttcctgaagcagtagagcagcccg) directed to the 3′ UTR region using methods previously described (38) with a hybridization temperature of 35° C and a wash temperature of 39° C. No hybridization was seen in nontransgenic littermates.

Immunohistochemistry (IHC). Brains were sectioned at 40 μm using a sliding freezing microtome, sections collected in cryoprotectant and stored at -20° C. Single and double-label free-floating IHC was performed using a rabbit polyclonal anti-human α-SYN Ab (1:7,500, Affiniti Research, Exeter, UK). This Ab is specific for hα-SYN protein as mouse α-SYN protein was not detected under the conditions used for IHC nor following Western blotting. The Alexa Fluor™ 594 goat anti-rabbit IgG fluorescent 2° Ab (1:750, Molecular Probes, Eugene, OR) was used. To identify mouse tyrosine hydroxylase (TH), a mouse monoclonal anti-TH Ab (Chemicon Int., Temecular, CA) was used (1:250) along with the Alexa Fluor™ 488 goat anti-mouse fluorescent 2° Ab (1:500, Molecular Probes, Eugene, OR). All sections were also incubated with 4′,6-diamidino-2-phenylindole (DAPI, 1:10,000, Sigma, St. Louis, MO) to identify nuclear DNA. All sections were coverslipped with Mowiol™ 4-88 (Polysciences, Inc., Warrington, PA). Slides were observed using either an Olympus Provis microscope or an Olympus Fluoview Personal Confocal microscope system (v1.2).

2-D polyacrylamide gel electrophoresis (PAGE) and Western blotting. Protein from mouse striata was subjected to two dimensional electrophoresis (31) by Kendrick Labs, Inc. (Madison, WI). Isoelectric focusing (IEF) was first carried out using 2% pH 4-8 ampholines (BDH ampholines, Gallard Schlesinger, Long Island, NY) for 9600 volt-hrs. One μg of an IEF standard, tropomyosin, was added and migrates as a doublet with the lower polypeptide spot of MW 33,000 and pI 5.2. After

equilibration in buffer "0" (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M tris, pH 6.8) each tube gel was sealed to the top of a stacking gel on top of a 10% acrylamide slab gel. After slab gel electrophoresis, the gel was placed in transfer buffer (12.5 mM Tris, pH 8.8, 86 mM glycine, 10% methanol) and electrophoretically transferred onto PVDF paper overnight at 200 mA and approximately 100 volts/two gels. The blot was stained with Coomassie Brilliant Blue.

The PVDF membranes were prewetted in 100% MeOH, transferred to Tris-buffered saline containing Tween (TBS-T, 20 mM Tris, pH 7.6, 137 mM NaCl, 0.1% Tween 20), blocked in TBS-T plus 5% milk and incubated for 3 hours in TBS-T plus 5% milk with an anti-α-SYN Ab (1:5,000, Chemicon Inc., Temeculah, CA) that recognizes both the mouse and human forms of α-SYN. Membranes were rinsed in TBS-T, incubated with an HRP-conjugated goat anti-rabbit 2° (1:5,000, Chemicon Inc., Temeculah, CA) for 1 hour, and then sequentially rinsed in TBS-T, TBS, and finally Tris-HCl (0.1 M, pH 8.6). Membranes were transferred to freshly prepared enhanced chemiluminescence solution containing 5.5 mg luminol, 0.28 mg p-coumaric acid, 7.7 µl of H_2O_2 (30%) in 25 ml Tris-HCl (0.1 M, pH 8.6) and gently agitated for 90 seconds. Membranes exposed to Kodak Biomax MR film.

Films were digitized using a CCD camera and analyzed using the 2-D gel analysis module of the MCID image analysis system (Imaging Research, Inc. St. Catherines, ON). The spot volume was determined as the area times the optical density.

Dopamine transporter (DAT) quantitative autoradiography (QAR). DAT autoradiography was performed and analyzed as previously described (37). Mouse sections were incubated with [^{3}H]-GBR 12935 (0.25 nM) and Zn^{2+}(50 µM). Films were exposed for 10 days.

High performance liquid chromatography (HPLC). HPLC was performed by either of two methods in two different labs depending on the age group of the animals. HPLC was always performed comparing transgenic mice with age-matched nontransgenic littermates. Values were

normalized to concurrent nontransgenic age-matched littermate controls. Dissected striata of adult mice at were homogenized in 0.1M $HClO_4$ containing 100 ng/ml 3,4-dihydroxybenzylamine (DHBA) as an internal standard. Homogenates were centrifuged, supernatants were filtered, and analyzed for levels of DA, 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) using high performance liquid chromatography with electrochemical detection (45). DA and metabolites were separated on a microbore reverse-phase column (C-18, 5 µm, 1x150 mm, Unijet, BAS) with a mobile phase consisting of 0.03 M citrate-phosphate buffer with 2.1 mM octyl sodium sulfate, 0.1 mM EDTA, 10 mM NaCl and 17% methanol (pH 3.6) at a flow rate of 90 µl/min and detected by a 6 mm glass carbon electrode (Unijet, BAS) set at +0.8 V.

Alternatively, striatal tissues were processed as previously described (43). Briefly, tissue was dissected and placed in 0.1M perchloric acid. The tissues were sonicated and centrifuged. The supernatants were stored at -80° C until analyzed for the concentrations of DA, DOPAC and HVA by HPLC - EC.. The pellets were later digested in 1 ml of 0.5N NaOH for measurements of protein concentration using Bio-Rad assay reagents. The concentrations of the neurotransmitters were expressed in terms of ng/mg protein. DA turnover was expressed as the ratio DOPAC/DA.

Behavioral assessments. Locomotor activity was quantified using automated locomotor activity chambers (Opto-Varimex Minor, Columbus Instruments International Corporation, Columbus, OH) as previously described (3, 43). Each chamber was equipped with infrared photobeams. Total photobeam breaks were recorded each minute for 45 minutes for horizontal, vertical, and ambulatory movements. Mice were initially habituated to the locomotor activity chambers in three 45-minute sessions (baseline activity) occurring on consecutive days, with all mice receiving vehicle i.p. injections prior to the session. Activity is presented as total counts.

After the third habituation session, some mice (nontransgenic littermate controls, lines hwα-SYN-5, and hm²α-SYN-39) were administered 15 mg/kg MPTP i.p. and motor activity assessed 1 hour, 24 hours and 5 days after each injection. MPTP injections were separated by one week. Two doses of MPTP were used. Animals were sacrificed six days after the second MPTP treatment.

After the third habituation session other mice (nontransgenic littermate controls, line hwα-SYN-5, and line hm²α-SYN-39) were administered 0.375 mg/kg amphetamine i.p. and the effects on motor activity assessed immediately afterwards (First Treatment). Amphetamine was repeated 7 days later and the effects assessed immediately afterwards (Second Treatment). In a separate experiment, mice (nontransgenic littermate controls, line hwα-SYN-88, and line hm²α-SYN-10) were treated with biweekly injections of amphetamine (1 mg/kg) and motor activity assessed immediately afterward. Mice received six injections biweekly and then received a rechallenge dose one week later.

The motor coordination screen was an inverted wire screen hanging test. Mice were placed individually on top of a square wire screen (13 x 13 cm of #4 mesh) mounted horizontally on a metal rod. The rod was then rotated 180°. The time taken to climb to the top of the screen was recorded with 2 min recorded if mice fell or remained clinging to underside of the screen. This test has demonstrated sensitivity to drugs such as haloperidol and results in fewer failures (e.g., falling or clinging) and less opportunity for incompatible interfering behavior than techniques such as the rotarod. Each mouse was tested on three separate trials at each session and the mean value of the last two trials was recorded.

Statistical analyses. Analysis of DA and metabolites were carried out with repeated measures analyses of variance (RMAONVA), with transgene status as a between-groups factor and neurotransmitter/metabolite as a within factor since these levels were derived from the same brain. Similarly, DAT densities were analyzed using RMANOVA with brain region as a within group

variable. This analytical approach provided conservative estimates. Motor activity level and inverted screen time across age were analyzed using RMANOVA with block (time) as a within-group factor and transgene status as a between groups factor. Total activity counts were compared with one-factor ANOVA using transgene status and age. Locomotor activity changes following MPTP treatment were also analyzed by RMANOVA across conditions (baseline-MPTP-recovery) and DAT density by RMANOVA with brain region as a within-factor. Significant effects were defined as $p \leq 0.05$. Significant effects determined by RMANOVA were followed by one-factor ANOVAs or Fisher's protected least significant differences test as appropriate.

Results

We created multiple lines of transgenic mice expressing either the wild-type hα-SYN (hwα-SYN) gene or a doubly-mutated (hm^{2}α-SYN) form of the hα-SYN gene containing both reported human mutations associated with the PDP (**Fig 1A**). Five founders from each construct were generated. Lines transmitting the transgene to offspring were characterized by Southern analysis, RT-PCR, QRT-PCR, ISHH, IHC, and 2-D PAGE followed by Western blotting. Digestion of genomic DNA using *Nco* I demonstrated a transgene specific fragment and mRNA expression was detected in the midbrain, eye, and adrenal gland using RT-PCR in all lines (data not shown). Lines hwα-SYN-5 and hm^{2}α-SYN-39 had higher expression of mRNA in the midbrain using QRT-PCR (data not shown) and ISHH (**Fig 1B**) compared to lines hwα-SYN-88 and hm^{2}α-SYN-10. High levels of hα-SYN protein were detected in the cell bodies of dopaminergic neurons in the midbrain (**Fig 1C**) and its main projection region the striatum (**Fig 1D**) in all lines. Nontransgenic littermate controls did not express transgene hα-SYN mRNA nor protein as assessed using IHC and a hα-SYN specific Ab (**Fig 1E**) nor by 2-D PAGE and Western blotting using a non-species specific α-SYN Ab (**Fig 1F**). In the brain of transgenic mice, catecholaminergic nuclei expressed both hα-SYN mRNA and protein. Labeling of hα-SYN protein was highest in the cell bodies and dendrites of SN and locus ceruleus (LC), their axons and terminal axon fields. The distribution of cells expressing the transgene and protein was consistent with others using this TH promoter (29, 41). The quantity of hα-SYN protein was measured in two lines from each construct using 2-D PAGE and Western blotting. The endogenous mα-SYN protein migrated as two spots with one larger isoform compared to a second very small isoform migrating at a slightly more basic pI and a slightly smaller mass and may represent a

dephosphorylation product (**Fig 1F**, 32). Both the hwα-SYN (**Fig 1H**) and hm²α-SYN (**Fig 1G**) protein migrated as single spots having slightly more acidic pIs than the endogenous mα-SYN protein and similar apparent masses. The amount of hα-SYN protein was always less than the amount of endogenous mα-SYN in the striatum. The percent of hα-SYN compared to mα-SYN (100% control) varied between the different transgenic lines (33.1 ± 8.9% in hwα-SYN-5, 41.3 ± 32.3% in hwα-SYN-88, 19.4 ± 10.2% in hm²α-SYN-10, and 48.8 ± 5.0% in hm²α-SYN-39). The fraction of hα-SYN to the total amount of αSYN (hα-SYN + mα-SYN) in the striatum of these constructs and lines was calculated (24.7 ± 5.1% in hwα-SYN-5, 27.3 ± 16.6% in hwα-SYN-88, 15.7 ± 7.5% in hm²α-SYN-10, and 32.8 ± 2.3% in hm²α-SYN-39; mean ± s.d., n = 2-4).

One line of each construct (hwα-SYN-5 and hm²α-SYN-39) was selected for further detailed study based on their high and comparable levels of hα-SYN mRNA expression in the SN, hα-SYN protein expression in the SN and striatum determined by IHC, and hα-SYN protein levels in the striatum determined by 2-D PAGE and Western blotting. Two additional lines (hwα-SYN-88 and hm²α-SYN-10) were examined to confirm and extend selected findings.

The localization of hα-SYN protein was compared to tyrosine hydroxylase (TH) using double-label IHC (**Fig 2**) in both lines of transgenic mice and in nontransgenic littermate controls. Both hα-SYN and TH (**Fig 2A-C**) were colocalized in cell bodies (**Fig 2D-F**) and dendrites of the SN, VTA and LC, their axons (**Fig 2G-I**), and in their terminal projection regions, the dorsal striatum (DS), ventral striatum (VS), and olfactory tubercle (OT) and cerebral cortex. The amount of hα-SYN protein varied among cells of the SNpc and VTA and was present in most cells that also expressed TH protein. The number of cells expressing the hα-SYN protein or the amount of protein per cell was greater in the VTA than in the SN. This corresponded to the amount of hα-SYN protein in projection regions which

was greater in the VS and OT than in the DS in both lines as visualized using IHC (**Fig 2B**). Whereas TH was strictly cytoplasmic in dopaminergic neurons (**Fig 2D and F**), hα-SYN was cytoplasmic and nuclear (**Fig 2E and F**) as originally described by others (24). Nuclear localized hα-SYN protein was absent in the nucleolus. In line hm^{2}α-SYN-39, the abnormal axons visualized in the median forebrain bundle (**Fig 2L**) were more dilated and beaded in appearance compared to those seen in nontransgenic littermate controls (**Fig 2J**). The terminals in the VS of mice from line hm^{2}α-SYN-39 also appeared abnormal (**Fig 2M**) with the smaller caliber processes and terminals often dilated or enlarged compared to those seen in nontransgenic littermate controls (**Fig 2K**). Neither compact round cytoplasmic inclusions nor intranuclear inclusions were identified in either transgenic line at 4 months of age. The cytoplasmic staining of TH and hα-SYN proteins was heterogeneous. In addition, the shape of dopaminergic neurons differed between line hm^{2}α-SYN-39 and both line hwα-SYN-5 and nontransgenic littermate controls suggesting a disturbance within the cell body.

The density of the DAT was determined in young mice using QAR in three regions (DS, VS, and OT) receiving dopaminergic input (**Fig. 3A**). The DAT density was increased in different subregions of the striatum in line hwα-SYN-5 (11-21%) and in line hm^{2}α-SYN-39 (12-23%) compared to nontransgenic littermates, although the small sample size might have limited the degree of significance (RMANOVA main effect of transgene status $F(2,9) = 3.395$, $p = 0.08$, $n = 4$ per group). Posthoc analysis of the effect of specific transgenes suggested again that the density of the DAT was greater in each transgenic line compared to nontransgenic littermate controls (Fisher's PLSD comparing line hwα-SYN-5 to nontransgenic littermates, $p = 0.08$ and line hm^{2}α-SYN-39 to nontransgenic littermates, $p = 0.04$). The elevated DAT density in the transgenic lines was greatest in the VS, with a 23% increase in line hm^{2}α-SYN-39 and a 21% increase in line hwα-SYN-5 consistent with the region expressing the greatest amount of hα-SYN protein visualized using IHC.

We next sought to determine if expression of hα-SYN altered the response to systemic amphetamine (**Fig 3B**). We habituated young mice to a locomotor chamber following i.p. injections of saline. We then injected mice with either saline or a low dose of amphetamine (0.375 mg/kg) i.p for two treatments separated by one week. RMANOVA revealed a significant interaction between lines of mice, treatment, and drug ($F(5,29) = 17.16$, $p < 0.0001$). There was no significant effect of amphetamine compared to saline after the first treatment ($F(5, 29) = 0.55$, **Fig 3B, left**). However, a significant effect of amphetamine after the second treatment occurred with revealing a significant interaction between line of mice and drug ($F(5, 29) = 16.7$, $p < 0.0001$, **Fig 3B, right**). A posthoc analysis showed significant increases in horizontal activity in both transgenic lines and nontransgenic littermate controls compared to saline (all ps < 0.05). The effect of amphetamine was significantly different in both transgenic lines compared to nontransgenic littermates, with line hwα-SYN-5 having a significantly greater response ($p < 0.02$) and line hm²α-SYN-39 having a significantly reduced response ($p < 0.003$) compared to nontransgenic littermate controls.

We then determined if the reduced sensitization-like response to amphetamine seen in line hm²α-SYN-39 was present in line hm²α-SYN-10 using a repeated dosage paradigm. We treated male nontransgenic littermate controls and lines hwα-SYN-88 and hm²α-SYN-10 with biweekly doses of amphetamine (1 mg/kg). The seventh dose of amphetamine (rechallenge dose) was given after a one week drug-free interval. There was a significant interaction between line of mice, treatment, and drug ($F(35, 574) = 1.695$, $p < 0.009$). Posthoc testing demonstrated that line hm²α-SYN-10 did not sensitize to amphetamine ($p > 0.05$) at the seventh dose in constrast to both nontransgenic littermate controls and line hwα-SYN-88 which did sensitize (both ps < 0.05).

We tested if vulnerability to MPTP neurotoxicity was altered in the transgenic lines. Young mice were habituated to an automated locomotor apparatus and then received two low doses of

MPTP (15 mg/kg) ip separated by one week with locomotor testing one hour, one day, and five days after each treatment. Both hα-SYN transgenic lines demonstrated enhanced behavioral sensitivity to MPTP (**Fig 4A**) and altered postmortem densities of the DAT (**Fig 4B**) following the second treatment with MPTP. Significantly greater decreases in locomotor activity (one hour after injection) compared to baseline were seen following both doses of MPTP in line hwα-SYN-5 (81% decrease from baseline, $p < 0.001$) and line hm^{2}α-SYN-39 (78% decrease from baseline, $p < 0.0001$) compared to nontransgenic littermates (34% decrease). Both lines hwα-SYN-5 line (11% decrease from baseline) and hm^{2}α-SYN-39 (21% decrease from baseline) exhibited an incomplete recovery in locomotor activity 24 hrs following the first dose of MPTP. The density of the DAT (**Fig 5B**) was reduced to a significantly greater extent following the two MPTP treatments in both transgenic hα-SYN lines (ANOVA main effect of transgenic status $F(2,30)=4.76$, $p=0.016$) compared to nontransgenic littermate control mice. The decline in DAT density was greatest in the VS and OT compared to the DS.

Finally, we examined age-dependent and gender effects of hα-SYN expression on two measures of motor behavior, spontaneous locomotor activity (**Fig 5A**) and the ability to right from an inverted position (**Fig 5B**). Neither transgenic line had differences in weight compared to nontransgenic littermates at any age. Analyses by gender did not demonstrate a significant gender effect in any group at any age, so genders were combined for both behavioral measures. Horizontal locomotor activity was measured at three ages, young age (2-3 months), middle age (7-9 months), and old age (13-23 months) and the inverted screen test was performed at two ages, young age (2-3 months) and old age (13-23 months). Both transgenic lines and nontransgenic littermates were always age-matched for both behavioral measures. In both behavioral assays, there was a significant effect of age, of transgene status, and an interaction between transgene and age (all p values < 0.05). At the young age, line hm^{2}α-SYN-39 was more active and had a shorter time to right than either line hwα-

SYN-5 or nontransgenic littermate controls (all p values < 0.05). Line hm²α-SYN-39 had a progressive and significant decline in locomotor activity (**Fig 5A**) at the middle ($p < 0.05$) and old ($p < 0.05$) ages compared to the young age which was matched by a significant increase in the inverted screen time in the old age group ($p < 0.0001$, **Fig 5B**) compared to the young age group. Both nontransgenic littermate controls and line hwα-SYN-5 had an age-related increase in the inverted screen time, but neither was significantly different from their young age group.

DA, HVA, and DOPAC were measured in the striatum at three ages, young age (2-3 months), middle age (7-9 months), and old age (13-23 months) using HPLC (**Fig 6A-C**). No gender-related differences were seen in any line for any measure. DA, DOPAC, and HVA levels were altered by age, transgenic status, and by an interaction ($F(7,108) = 6.7$, $p < 0.0001$; $F(7,108) = 4.9$, $p < 0.0001$; $F(7,108) = 4.7$, $p < 0.0001$, for DA, DOPAC, and HVA respectively). Posthoc analyses revealed the primary change to occur in line hm²α-SYN-39, where an age-related decline was seen primarily in the old age group for all three measures (all ps < 0.001) compared to nontransgenic littermates and line hwα-SYN-5 (**Fig 6A-C**). The locomotor changes seen in line hm²α-SYN-39 at a young age were associated with a decrease in DA levels and a normal level of DOPAC producing a significantly increased ratio of DOPAC/DA ($p = 0.02$). At the old age the ratio of DOPAC/DA was not altered due to similar levels of decline.

DA, HVA, and DOPAC were measured in the striatum of lines hwα-SYN-88 and hm²α-SYN-10 at 7-9 months of age. Compared to both nontransgenic littermate controls and line hwα-SYN-88, line hm²α-SYN-10 had 27-31% lower levels of DA (both posthoc ps < 0.05) and HVA (both posthoc ps < 0.05). Levels of DOPAC were preserved in line hm²α-SYN-10 at this age. These changes are similar to those seen in line hm²α-SYN-39 at this age. Line hwα-SYN-88 did not differ in any measure from nontransgenic littermates similar to line hwα-SYN-5 examined at both young and old ages.

Discussion

The hα-SYN protein expressed in the transgenic mice reported here is biologically active in terminals of the dopaminergic nigrostriatal pathway as demonstrated by an increased density of the DAT, altered locomotor activity responses to amphetamine, and increased sensitivity to MPTP. The biological effect of hα-SYN protein differed between the hwα-SYN and hm^{2}α-SYN forms with the hm^{2}α-SYN displaying adverse effects including altered morphology of DA cell bodies and processes, reduced locomotor response to amphetamine, loss of amphetamine-induced sensitization, increased motor activity at a young age, progressive motor impairments with increasing age, and age-related alterations in DA and metabolite levels. These findings have significant implications for understanding the normal role of α-SYN in DA terminals and the role of hm^{2}α-SYN in contributing to the PDP.

Physiologic effects of hα-SYN expression. The specific function(s) of α-SYN remains to be determined. The cellular localization in terminals and other studies suggest a role in presynaptic function (6, 30). α-SYN has been shown to interact with a variety of proteins including synphilin-1 and TH (8, 34) and a functional interaction with the DAT has been demonstrated (22). We demonstrate that expression of hα-SYN in transgenic mice resulted in increased density of the DAT. The number of neurons expressing the hα-SYN protein or the amount per cell was greater in the VTA compared to the SNpc resulting in a greater amount of protein in the VS and OT compared to the DS as visualized by IHC, which might have contributed to the greater increase in density of the DAT in the VS and OT than in the DS. Finally, the toxicity of MPTP, as determined by the magnitude of the decrease in regional DAT levels, was greater in the VS and OT than in the DS in transgenic mice.

These finding can be experimentally verified by further examination of other lines of hα-SYN transgenic mice expressing either reduced or increased amounts of hα-SYN or lines with different levels of regional expression. A dose-response effect may be important to relate to humans where the concentration or activity of hα-SYN protein may be a predisposing factor in the PDP.

Expression of hα-SYN in these transgenic mice resulted in a variety of functional effects, which might relate to interactions with the DAT or other synaptic proteins. hwα-SYN expression in transgenic mice resulted in an increased sensitization-like response to a low dose of amphetamine on a second administration (line hwα-SYN-5). The low dose used was reported to not have an effect following the first administration, but resulted in sensitization on further injections (27). Sensitization occurred after repeated doses of amphetamine in line hwα-SYN-88. These results are consistent with the increased response to amphetamine seen in rats transduced with a hwα-SYN expressing virus and the decreased response to amphetamine seen in α-SYN knockout mice (1, 19).

A functional role for hwα-SYN expression was also demonstrated by increased sensitivity to MPTP, which might relate to it's interaction with the DAT since the active metabolite of MPTP, MPP^+, is taken into DA terminals via the DAT (7, 12). Expression of hα-SYN in transgenic mice may also exacerbate MPTP toxicity via another mechanism. We tested only two small doses of MPTP and were unable to detect any differences between hwα-SYN and hm^{2}α-SYN transgenic lines. However, larger or more doses, or different ages might demonstrate a greater susceptibility in the hm^{2}α-SYN lines compared to the hwα-SYN lines.

Pathologic effects of hm^{2}α-SYN. Expression of hm^{2}α-SYN demonstrated several differences from hwα-SYN suggesting it may be toxic. Differences included morphologic abnormalities in cell bodies and processes and age-related alterations in behavior likely reflecting the changes in DA and

metabolites. The behavioral differences between hwα-SYN and hm²α-SYN lines were dramatic. At the youngest age examined, line hm²α-SYN-39 demonstrated significantly increased locomotor activity and a shorter time to right compared to line hwα-SYN-5 and nontransgenic littermates. These behavioral effects seen in line hm²α-SYN-39 were associated with reduced DA levels and increased DA turnover, suggesting these behavioral effects might be related to abnormal DA homeostasis in terminals. These changes are suggestive of increased or abnormal DA release resulting in increased metabolism or turnover. Locomotor dysfunction in line hm²α-SYN-39 was observed with aging, leading to a parkinsonian phenotype of bradykinesia and hypokinesia. This phenotype was associated with further reductions in DA levels and significant reductions in DOPAC and HVA.

Expression of hm²α-SYN also impaired locomotor responses to amphetamine. Locomotor response to a second low dose of amphetamine was significantly reduced in line hm²α-SYN-39 and sensitization to repeated higher doses of amphetamine was blocked in line hm²α-SYN-10. These findings suggest an important role for hα-SYN in regulating responses to repeated doses of amphetamine and are consistent with the location of α-SYN in terminals. This effect may be related to the DAT or other proteins interacting with α-SYN in DA terminals.

Line hm²α-SYN-39 had an abnormal appearance to neuronal cell bodies and processes. The axons in this line were similar to those seen in human postmortem PD tissue (2, 13). Others have also reported abnormal neurites in hα-SYN transgenic models (10, 25, 44). In contrast to other models, only line hm²α-SYN-39 appeared to produce these morphological effects in our lines. However, we have not yet developed a quantitative or objective methods to assess these changes and normal dopaminergic axons are beaded and have punctate terminals.

Both intracytoplasmic (10, 25) and intranuclear (25) inclusions have been identified in other transgenic models expressing α-SYN. We did not identify either type of inclusion in our mice. This may relate to strong cytoplasmic staining of hα-SYN obscuring the presence of inclusions, the young age we have examined so far, the limited number of neurons examined, or the fact that we have expressed hα-SYN in cells also expressing mα-SYN which may act to reduce the tendency for hα-SYN to spontaneously aggregate. This was demonstrated by *in vitro* aggregation studies combining mα-SYN and hα-SYN (39). High levels of hα-SYN in cells not expressing α-SYN may be more likely to aggregate. This is a testable hypothesis *in vivo*.

Others have reported adverse properties of hα-SYN in a variety of model systems ranging from cell culture (33) to transgenic flies (10) and mice (25, 44). Both the wild-type and mutated form of hα-SYN appear to be toxic in these studies, with some studies suggesting greater toxicity with mutant forms (33). Although the mutant forms of hα-SYN are toxic in humans because of their association with inherited forms of the PDP; the role of wild-type α-SYN in causing neurodegeneration has been less clear. The ability of α-SYN to spontaneously aggregate *in vitro* and *in vivo* under physiologic and altered environments suggests this may be a leading factor in the detrimental effects of overexpressed α-SYN (11). The widespread ability of this protein to be toxic in a variety of cells and conditions suggests some cells may not tolerate this gene product. The restricted location of α-SYN expression in the CNS suggests that transgenic or ectopic expression in other neuronal groups may be adverse. Data from our study suggest that hwα-SYN was not directly toxic to SN neurons, at least up to 18 months of age in the absence of exogenous neurotoxins and did not form aggregates as described by others or to the degree that was seen with expression of $hm^2\alpha$-SYN. There are a variety of explanations for the discrepancy between our transgenic mice and other studies

demonstrating either a toxic role for hwα-SYN or the absence of a toxic role with mutated forms of hα-SYN (26, 36). One may relate to the amount of protein expression. This is particularly important for a protein with a tendency to aggregate in a concentration-dependent manner such as α-SYN. The stoichiometric relationship between transgene product and endogenous gene product may be most relevant in the *in vivo* setting. In the transgenic lines reported here, the level of hα-SYN accounted for ~25 to 33% of the total amount of α-SYN (hα-SYN plus mα-SYN) present in the mouse striatum. We presume that at this stoichiometric ratio hwα-SYN is insufficient to produce a pathologic phenotype. Another difference between studies is the transcriptional control of the transgene. In our mice the TH promoter was used to direct expression to catecholaminergic neurons. Others employed promoters yielding widespread expression. This is in contrast to the normally restricted regional expression in rodents (16). This raises the possibility that forced expression of hwα-SYN in cells not normally expressing it may result in toxicity. A third difference between models is the strain background. Considerable data have shown marked effects of strain background on a variety of genetically engineered genotypes and following neurotoxic exposure (14, 9, 5). Another difference is our use of a doubly-mutant form of hα-SYN which may have unique properties compared to the two single mutations. Irrespective of which of these considerations may apply it is clear that TH promoter driven hm^{2}α-SYN, but not hwα-SYN elicits adverse effects in the nigrostriatal system in these lines. Future studies will aid in sorting out these differences among models and hopefully shed light on the human condition.

Model and mechanisms. A model for the role of hα-SYN in neurodegeneration may be emerging. The subcellular location of hα-SYN suggests it may have more than one function, but it clearly plays a role in presynaptic terminals. One role may include regulating the density or activity of the DAT. Alterations in the amount or function of the DAT may have significant effects on

dopaminergic neurons, either via dopamine (22), its metabolites, or via neurotoxicants. Modest increases in mouse striatal DAT (20-30%) may increase significantly the response to MPTP (> 50% loss of neurons, Donovan et al., 1999). Likewise, reductions in the DAT reduce the toxic response to MPTP (12). Alternatively, dysfunction of hα-SYN due either to a mutation, the amount of protein, aging, or an interaction with environmental toxins may lead to altered function either as aggregates or as a toxic interaction with another protein. This may result in impaired axonal transport and/or terminal dysfunction recognized by dystrophic neurites or impaired DA terminal metabolism. Affected DA terminals would be predicted to be more susceptible to oxidative injury. The observed increases in the DAT may over time contribute to presynaptic dysfunction through accelerated and prolonged energy-consuming reuptake of DA.

The mechanism for nigral neurodegeneration resulting from mutations in hα-SYN is most consistent with a gain of toxic function. The behavioral, anatomical and neurochemical observations in the hwα-SYN and hm^{2}α-SYN lines suggest a possible pathogenic sequence. In young adult mice expression of hwα-SYN is sufficient to elicit presynaptic DAT upregulation without perturbing DA metabolism. Expression of the hm^{2}α-SYN at the same age results not only in presynaptic DAT upregulation, but also adversely affects DA metabolism and responses to amphetamine. We speculate that nigrostriatal presynaptic dysfunction is achieved in part by a hα-SYN mediated increase in DAT activity and attendant increased presynaptic metabolic activity associated with neurochemical dysregulation. Furthermore, we posit that the hm^{2}α-SYN form of the protein is pathogenic as it continues this effect over time resulting in progressive failure of DA terminals. Others have provided evidence linking an association between the levels of the DAT and the vesicular monoamine transporter (VMAT2) in the pathogenesis of toxin-induced and idiopathic PDP

(28) suggesting an important role for the DAT. However, other proteins interacting with α-SYN must still be considered.

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FIGURE LEGENDS

Fig. 1. Generation and characterization of hα-SYN transgenic mice. (**A**) Map of the pUTHTV/hm^{2}α-SYN construct. Sites for PCR primers used to screen genomic DNA and amplify the original hα-SYN cDNA (**1**), sites for PCR primers used for RT-PCR to identify mRNA/cDNA and appropriate splicing of the construct (**2**), real-time QRT-PCR primers (**3**) and probe (**3**) for detecting and quantifying mRNA, and the site of the 48 mer oligonucleotide probe (**4**) used for ISHH. (**B**) Image of ISHH from line hm^{2}α-SYN-39 at the level of the SN. (**C, D, E**) IHC using a human specific polyclonal Ab to hα-SYN in line hm^{2}α-SYN-39 (**C, D**) or a nontransgenic littermate control (**E**). This Ab detected only hα-SYN protein (**C, D**), but not mα-SYN (**E**). Human α-SYN protein was most highly expressed in the cell bodies and dendrites in the SN and VTA, their axons, and in the striatum (**D**). Striatal protein was subjected to 2-D PAGE followed by Western blotting using a polyclonal Ab to α-SYN that recognizes both mouse (mα-SYN) and human (hα-SYN) α-SYN. (**F**) Nontransgenic littermate controls demonstrated a larger single spot migrating at approximately 15 kDa and a much smaller spot (arrow) migrating at a slightly smaller MW and at a slightly more basic pI suggestive of a dephosphorylated form of mα-SYN. Lines hm^{2}α-SYN-39 (**G**) and hwα-SYN-5 (**H**) demonstrated unique spots migrating at a similar MW to mα-SYN, but at different and slightly more acidic pIs.

Fig 2. Colocalization of hα-SYN protein and abnormal axons and neurites in young hα-SYN transgenic mice. Double-label fluorescent IHC for TH (**A, D, G, green**), hα-SYN (**B, E, H, Red**), both hα-SYN and TH (**C, F, I**) in a sagittal section from line hm^{2}α-SYN-39 (**A-C**), or a line hwα-SYN-5 SN neuron (**D-F**), or from a line hm^{2}α-SYN-39 mouse median forebrain bundle (MFB, **G-I**). Confocal microscopy was used to image sections at 0.4 μm thickness using either the red or green filter (**D-I**) or

routine fluorescent microscopy at 1.25 x (**A-C**). The hα-SYN protein was present in dopaminergic cell bodies and dendrites (**B and E**) and nigrostriatal axons and terminals (**B and H**) in a distribution that matched that of TH (**A, D and G**). The hα-SYN protein was present in both the cytoplasm and the nucleus of neurons (**E**). Abnormal neurites were present in dendrites of SN and VTA neurons and axons to the striatum (**G-I**) and elsewhere. Fluorescent IHC for TH was performed in a nontransgenic littermate control mouse (**J and K**) or a mouse from line hm²α-SYN-39 (**L and M**) in the MFB (**J and L**) or the VS (**K and M**). Confocal microscopy using a 100X objective at 0.5 μm steps was performed and a total of 10 sections stacked to make the final image. TH is normally present in MFB axons of control mice in a discontinuous manner with the majority of axons having a uniform diameter with occasional modest dilations (**J**). In hm²α-SYN-39 transgenic mice TH axons were more beaded and dilated in appearance with more discontinuities (**L**). In the VS, TH was present in smaller caliber processes and terminals of control mice (**K**). In hm²α-SYN-39 transgenic mice the smaller caliber processes and terminals were more dilated and punctate (**M**).

Fig. 3. Density of the dopamine transporter (DAT) and locomotor response to amphetamine in young hα-SYN transgenic mice. (**A**) Both transgenic lines (hwα-SYN-5 and hm²α-SYN-39) demonstrated modest, but significant increases (10-20%) in the density of the DAT that varied by line and region. (**B**) Mice were given either saline or amphetamine (0.375 mg/kg) i.p. and assessed for locomotor activity over a 45 min test session (**First Treatment**). The treatment was repeated seven days later (**Second Treatment**). RMANOVA revealed a significant interaction between lines of mice, treatment, and drug ($F(5,29) = 17.16$, $p < 0.0001$). Posthoc testing revealed no significant differences between lines of mice or drug during the first treatment ($F(5, 29) = 0.55$). Posthoc testing revealed significant interactions between line of mice and drug on the second treatment ($F5, 29 = 16.7$, $p <$

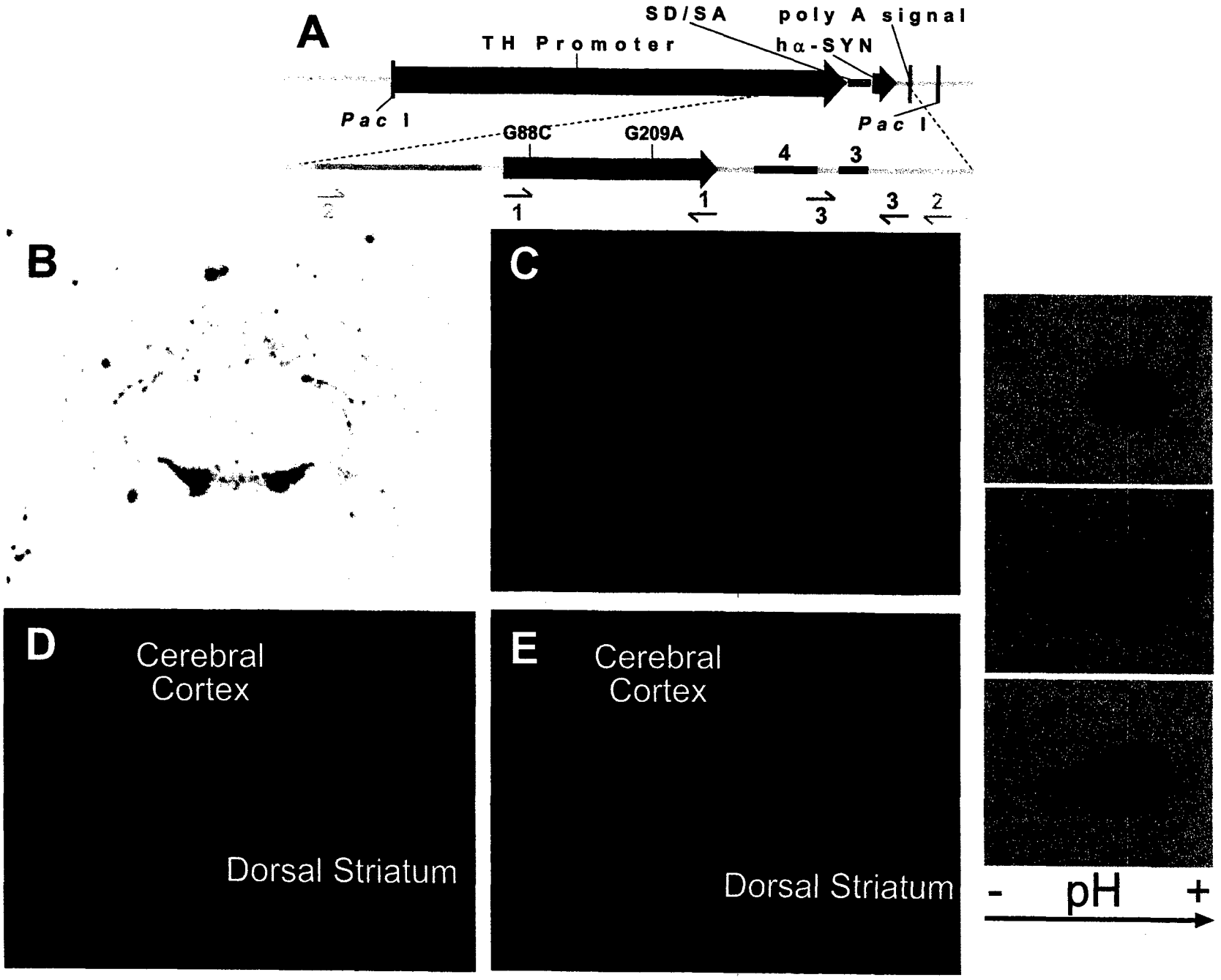
0.0001). [a, b, c]Significant effect of amphetamine compared to saline were found in all three lines on the second treatment (all p values < 0.05). Both lines [c]hm²α-SYN-39 ($p < 0.03$) and [b]hwα-SYN-5 ($p < 0.02$) were significantly different from nontransgenic littermate controls and from each other ($p < 0.0001$).

Fig 4. Effects of MPTP on young hα-SYN transgenic mice. (**A**) Locomotor activity as percentage of score in mice prior to MPTP (**baseline**), one hour (**MPTP**), and five days after MPTP (**recovery**). Both transgenic lines demonstrated significantly greater reduction in locomotor activity one hour after MPTP compared to nontransgenic littermate controls (◀, $p < 0.001$). (**B**) The density of the DAT (reported as the % of the untreated value in each line for each region) decreased in both lines of transgenic mice to a greater extent than in nontransgenic littermate controls after the two low doses of MPTP (ANOVA main effect of transgenic status $F(2,30)=4.76$, $p=0.016$).. The decreases varied by region and line.

Fig. 5. Age-related behavioral measures. (**A**) Horizontal activity was measured for each group at three ages, young age (2-3 months), middle age (7-9 months), and old age (13-23 months). RMANOVA demonstrated a significant effect of transgenic status ($F(2, 286) = 11.0$, $p < 0.0001$), age ($F(2, 286) = 17.3$, $p < 0.0001$), and an interaction between group and age ($F(4, 286) = 11.5$, $p < 0.0001$). [a]Individual post hoc analyses demonstrated that at each age, line hm²α-SYN-39 was significantly different from nontransgenic controls and line hwα-SYN-5 (all p values < 0.05). Line hm²α-SYN-39 demonstrated a progressive age-related decline with both the middle and old age groups significantly different from the young age group (both p values < 0.0001). (**B**) Time to right from an inverted screen was measured for each group at two ages, young age (2-3 months) and old age (13-23

months). RMANOVA demonstrated a significant interaction of transgenic status and age ($F(2, 181) = 4.6$, $p = 0.01$). [a]Individual post hoc analyses demonstrated that line hm^{2}α-SYN-39 at a young age was significantly different from both nontransgenic littermate controls and line hwα-SYN-5 (both ps < 0.03). [b]Individual post hoc analyses demonstrated that line hm^{2}α-SYN-39 at an old age was significantly compared to a young age ($p < 0.0001$).

Fig. 6. Age-related striatal neurotransmitter levels. HPLC data for DA (**A**), DOPAC (**B**), and HVA (**C**) from striatum were obtained at three ages: young age (2-3 months), middle age (7-9 months), and old age (16-18 months) except for line hwα-SYN-5 in which values were obtained only at the young and old ages. The HPLC data were collected in two different labs using different procedures and equipment. However, nontransgenic littermate control mice were always included in each assay. All values were normalized to the mean of the nontransgenic littermates at the appropriate age (error bars indicate SEM). The absolute values (mean ± SEM in units of ng/mg wet tissue weight or in units of ng/mg protein) for the nontransgenic littermate group from the two labs: DA (7.59 ± 0.29; 121.5 ± 6.63), DOPAC (1.04 ± 0.06; 8.92 ± 0.56), HVA (1.01 ± 0.08; 8.18 ± 0.51). Levels of DA and DA metabolites in line hwα-SYN-5 were similar to those of nontransgenic littermates at the two ages examined. RMANOVA demonstrated a significant effect of transgene, age, and an interaction for each measure (all p values< 0.05). Posthoc analyses revealed a significant decline in all three measures in line hm^{2}α-SYN-39 at the old age compared to nontransgenic littermates and line hwα-SYN-5 (all p values < 0.0002).


A
SD/SA
poly A signal
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Pac I
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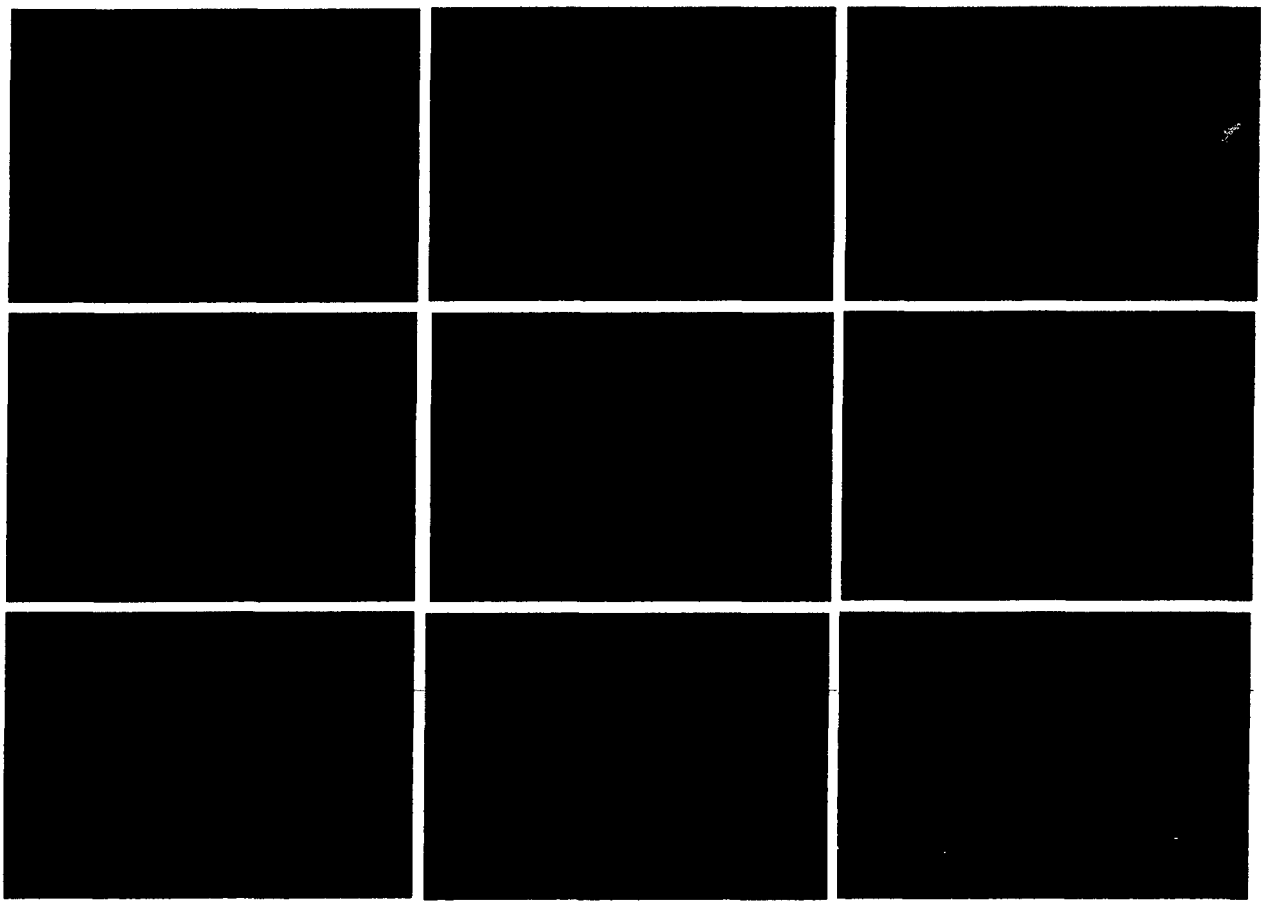
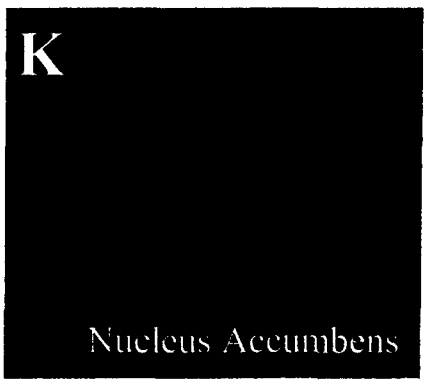
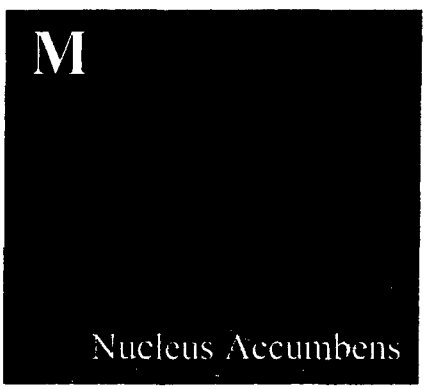



J
Median Forebrain Bundle


K
Nucleus Accumbens


L
Median Forebrain Bundle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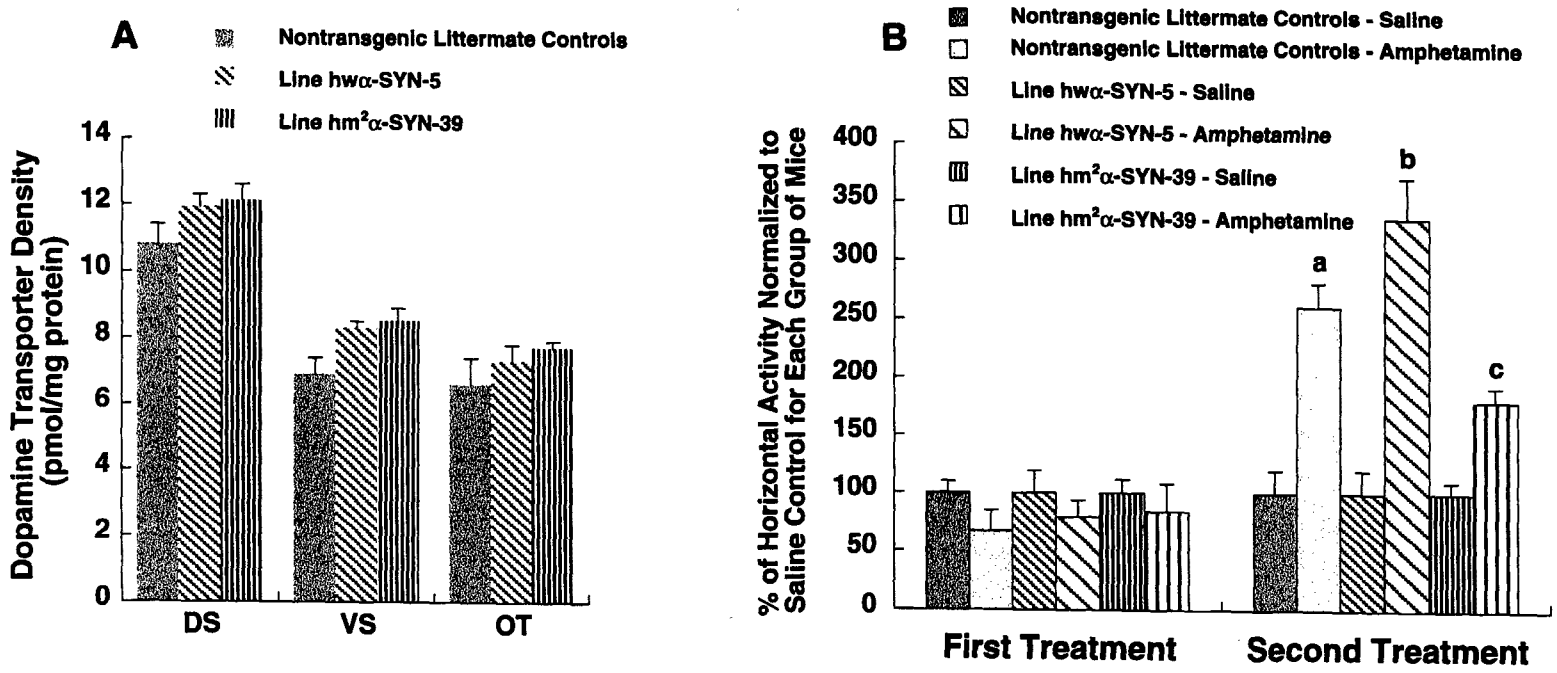

Fig 3 Top ↑
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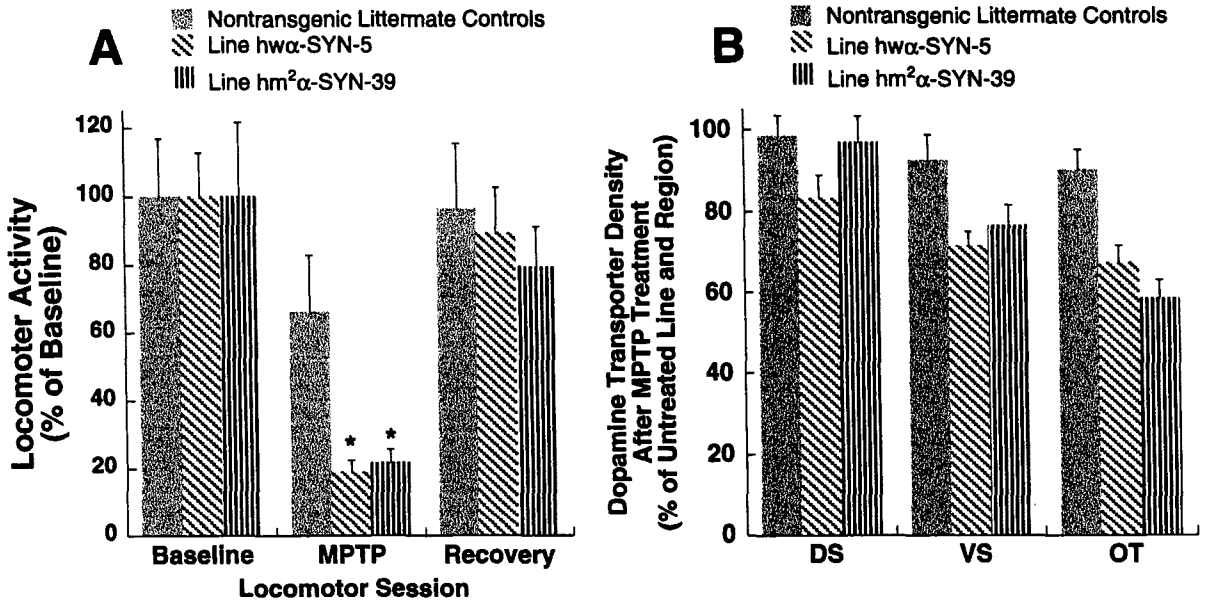

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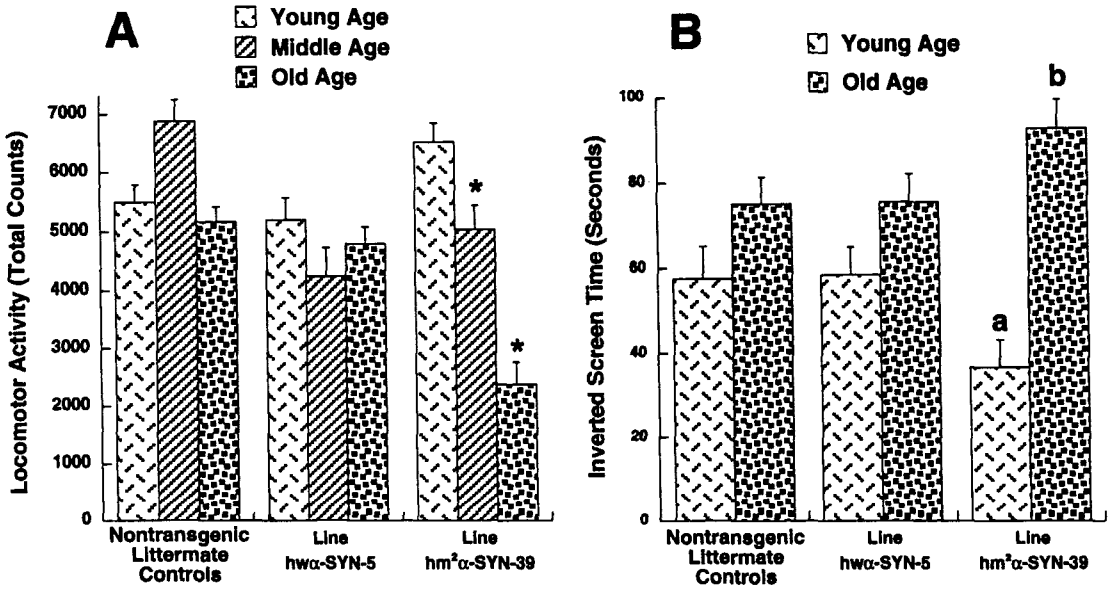

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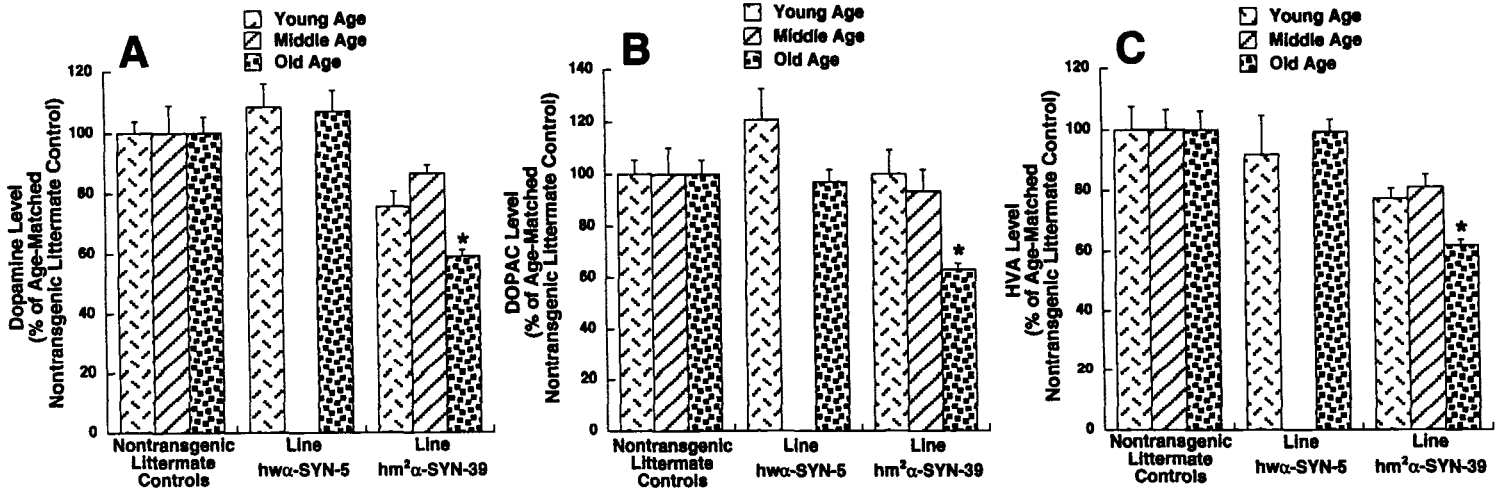

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